Pro Gradu

The effects of hypoxia on gestational diabetes mellitus in mice

Florian Mohr

University of Oulu

Faculty of Biochemistry and Molecular Medicine2020

Place of experimental work: Karppinen Group

Supervisor: Peppi Karppinen

Abbreviations

Abbreviation	Description
aa	Amino acid
ADM	Adrenomedullin
ARNT	Arylhydrocarbon receptor-nuclear translocator
ATP	Adenosine triphosphate
AUC	Area under the curve
bHLH	Basic helix-loop-helix
BL	Baseline
BMI	Body mass index
C	Celsius
CAD	Carboxy activation domain
Ccl2	Chemokine ligand 2
CCNG2	Cbp/p300-interacting transactivator
CHER	Cholesterol esterase
CHOD	Cholesterol oxidase
CoA	Coenzym A
CODDD	C-Terminal-oxygen-dependent degradation domain
CP	Ceruloplasmin
CTAD	C-terminal transactivation domain
CXCR4	Chemokine receptor 4
D	Aspartate
DEC1	Differentiated embryo chondrocyte 1
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
ER	Endoplasmic reticulum
ET1	Enothelin 1
FFA	Free fatty acid
FIH1	Factor inhibiting HIF1α
FN1	Fibronectin 1
g	Gram
GAPDH	Glyceraldehyde-3-phosphate
GDM	Gastational diabetes mellitus
GLUT-1	Glucose transporter 1
GLUT-4	Glucose transporter 4
GTT	Glucose tolerance test
H	Histidine
h	Hours
H	Hypoxia
Hb	hemoglobin
HDL	High density lipoprotein
HE	Hematoxylin-eosin
HIF	Hypoxia inducable factor
HIF-p4Hs	HIF prolyl 4-hydroxlases

HOMA-IR	Homeostatic model asssement-insulin resistance
HREs	Hypoxia response elements
HRP	Horseradish peroxidase
IADPSG	International Association of Diabetes in Pregnancy Study Groups
IGF2	Insulin like growth factor
iNOS	Inducible nitric oxide synthase
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
IRβ	Insulin receptor β-subunit
IUGR	Intrauterine growth restriction
K	Lysine
kDA	Kilodaltons
l	Liter
LDHA	Lactate dehydrogenase
LPL	Lipoprotein lipase
LRP1	LDL receptor related protein 1
mg	Milligram
min	Minutes
ml	Milliliter
mmHg	Millimeter of mercury
mmol	Milimole
MMP2	Matrix metalloproteinase 2
mRNA	Messenger RNA
N	Normoxia
NAFLD	Non-alcoholic fatty liver disease
NC	Normal chow
NEFA	Non-esterified fatty acids
nm	Nanometer
NODDD	N-terminal-oxygen-dependent degradation domain
NTAD	N-terminal transactivation domain
OD	Obesogenic diet
ODDD	Oxygen-dependent degradation domain
OXPHOS	Oxidative phosphorylation
P4H	Prolyl 4-hydroxlase
P4H-TM	Transmembrane P4H
P4HA1	Collagen propyl 4-hydroxylase alpha 1
PAI1	Plasminogen activator inhibitor 1
PAS	Per-ARNT-Sim
PCR	Polymerase chain reaction
PDK1	Pyruvate dehydrogenase lipoamide kinase isozyme 1
PfkI	Phosphofructokinase 1, liver type
pmol	Picomole
pVHL	Von Hippel-Lindau protein
qPCR	Quantitative realt-time PCR
R	Arginine
RNA	Ribonucleic acid

rpm	Revolutions per minute
s	Seconds
SEM	Standard error of mean
TAD	Transactivation domain
TFR	Transferrin receptor
TG	Triglyceride
TGFα	Transforming growth factor alpha
TGFβ3	Transforming growth factor beta 3
TIC	Transcription initiation complex
TMP	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TWIST1	Twist-related protein 1
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VEGFR1	Vascular endothelial growth factor receptor 1
VHL	Von Hippel-Lindau
VLDL	Low-density lipoproteins
WAT	White adipose tissue
WHO	World Health Organization
µg	Microgram
µl	Microliter
IUGR	Intrauterine growth restriction
NAFLD	Non-alcoholic fatty liver disease

Contents

1 LITERATURE SECTION

1.1 Introduction

Hypoxia and oxygen sensing are mechanisms which play important roles in many physiological processes like the generation of new blood vessels, production of red blood cells, fetal development as well as in pathophysiological processes like chronic renal failure and cancer. Especially since the Nobel Prize in Physiology or Medicine 2019 was awarded to William G. Kaelin, Gregg L. Semenza and Peter J. Ratcliffe working on this specific topic a broader range of people became aware of its importance. (The Nobel Prize in Physiology or Medicine 2019 - Press release n.d.). Our body is reacting to decreased oxygen by activation of the hypoxia response which is mediated by special transcription factors the so-called hypoxia inducible factors (HIFs). Under hypoxic conditions the HIFα subunit is able to translocate to the nucleus and form there a dimer with the HIFβ subunit and trough that activating hypoxia related genes. In the presence of oxygen HIF prolyl 4-hydroxylases (HIF-P4Hs) hydroxylates the HIFα subunits which is leading to proteasomal degradation and therefore no activation of hypoxia related genes (Kaelin and Ratcliffe 2008).

Hypoxia also plays an important role in pregnancy. Worldwide over 140 million people live in high altitude (> 2500 m above sea level) and therefore many pregnant women facing oxygen restriction during pregnancy. High altitude has a strong impact on the birthweight of the child, which is reduced around 100 g every 1000 m above sea level (E. Krampl 2002)(Soria et al. 2013). This is caused by changing the glucose availability for the fetus. More glucose is utilized by maternal tissue instead of directed to the growing fetus (Elisabeth Krampl et al. 2001).

Gestational diabetes mellitus (GDM) is an alteration during pregnancy with a high prevalence many women facing worldwide with ranging percentages from 6.1 to 30 % (McIntyre et al. 2019). It is a state hyperglycemia which develops during pregnancy which can range from mildly impaired glucose tolerance to glucose levels which are common in diabetic patients (Boyd E. Metzger 2010). Those elevated blood glucose levels lead to short- and long-term consequences for the mother and child which include fetal overgrowth and pre-eclampsia. Children of mothers with GDM have also an increased risk of developing the metabolic syndrome, pre-diabetes and type 2 diabetes in later life (Harder et al. 2001)(Aerts and Van Assche 2006).

1.2 Review of the Literature

1.2.1 Hypoxia response Pathway

1.2.1.1 Hypoxia

Hypoxia describes a condition, which is defined as the failure of oxygenation at tissue level; this means that there is not enough oxygen supply to sustain oxidative phosphorylation (OXPHOS) (Samuel and Franklin 2008). In order to maintain cellular oxygen homeostasis different oxygen concentrations are required in different tissues. For instance in lung alveoli 14.5 % O_2 and in peripheral tissues 3.4-6.8 % O_2 are needed (Carreau et al. 2011). Hypoxia can be caused by different reasons and has therefore different forms. Those forms are defined by the localization of hypoxia, whether the failure of oxygenation is on a local level or the whole system lacks oxygen supply. Local forms of hypoxia can be caused e.g. by ischemia which is the insufficient supply of blood to a certain tissue or a lack of vascularization like in bigger fat deposits of obese people (Andrei et al. 2017). An example of a systemic lack of oxygen is called hypoxic hypoxia. This form of hypoxia occurs when the lungs are not able to fully oxygenate the blood which can be caused by pulmonary diseases or is also developed at high altitudes (> 2500m) (Forms of hypoxia n.d.).

1.2.1.2 Life with and without oxygen

Oxygen changed the life of eukaryotic organisms profoundly around 1.5 billion years ago. At this time organisms which contained mitochondria through symbiogenesis appeared for the first time on Earth. Those organisms were capable of oxidizing glucose to CO_2 and water in the mitochondria through OXPHOS. That process highly relies on O_2 as the final electron acceptor and cannot take place without it. In the absence of O_2 the organism has to produce its energy mostly through glycolysis and lactic acid fermentation. In comparison to glycolysis, OXPHOS produces 18 times more ATP per mole glucose. This step of energy production was crucial for the evolution of metazoan and every multicellular organism highly depends on this kind of energy supply, for which a constant flow of O_2, the oxygen homeostasis is crucial (Semenza 2007).

For the oxygen homeostasis oxygen sensing mechanisms are necessary and have evolved in metazoan organisms. Higher organisms have several ways to sense the oxygen concentration in their systems. Mammalians for example possess so called carotid bodies for measuring the oxygen content of the arterial blood (Weir et al. 2005). A lower O_2 concentration in the blood

would be counteracted by increasing the heart rate as well as the respiration as an acute response of the organism (Heinonen, Boushel, and Kalliokoski 2016). However, there is one mechanism which is present in all metazoan organisms, the so-called hypoxia response pathway. Its main component is the master regulator of oxygen homeostasis, the hypoxia-inducible factor (HIF) (Semenza 2007).

1.2.1.3 Hypoxia-inducible factor (HIF)

HIF is a transcription factor which promotes the expression of over hundreds of genes in a low oxygen environment (Semenza 2012). Structure wise it is a heterodimer of an oxygen-sensitive HIFα and a constitutively expressed HIFβ subunit and belong to the basic helix-loop-helix (bHLH)/Per-ARNT-Sim (bHLH/PAS) family of transcription factors. In absence of oxygen HIFα accumulates in the cytosol and translocates to the nucleus to form a heterodimer with HIFβ (Song et al. 2008). The dimer binds the DNA at hypoxia response elements (HREs) which are containing the core sequence (G/ACGTG) and activates the transcription of multiple hypoxia related genes (Schofield and Ratcliffe 2004). The HIFα-subunit has 3 known isoforms: HIF1α, HIF2α and HIF3α, whereby HIF3α has multiple splice variants and is not likely to act as a transcription factor (Pasanen et al. 2010). It is thought to be a regulatory element through inhibiting the hypoxia pathway in different ways (Schofield and Ratcliffe 2004).

HIF1α and HIF2α are the most extensively studied isoforms. Human HIF1α is 826 and HIF2α 870 amino acids (aa) long and share 48 % of the aa sequence but also show structural and biochemical similarities (Ke and Costa 2006). Both contain two transactivation domains, the N-terminal (NTAD) and the C-terminal (CTAD) as well as Per-ARNT-Sim homology (PAS) domain and the previous mentioned bHLH which are responsible for DNA binding and dimerization with the β-subunit.

In contrast to the HIF β-subunit which is stable and also known as ARNT (arylhydrocarbon receptor-nuclear translocator), the α-subunits are highly unstable and their degradation depends on the oxygen level in the cell (Kaelin and Ratcliffe 2008). For regulation HIFα subunits have two oxygen-dependent degradation domains (ODDD), the N-terminal-and the C-terminal-ODDD (NODDD and CODDD) with proline residues for hydroxylation (Fig. 1) (Schofield and Ratcliffe 2004).

The expression patterns of HIF1α and HIF2α differ from each other. HIF1α seems to be expressed in all cell types whereas HIF2α expression is more restricted. Only specific cell types like endothelial cells, glial cells, cardiomyocytes, kidney fibroblasts, interstitial cells of the

pancreas and duodenum and hepatocytes show HIF2α expression (Zhao et al. 2015). The mRNA levels for HIF2α are particularly high in lung, heart and endothelium tissues which are important for the systemic oxygen delivery (Tian, McKnight, and Russell 1997)(WIESENER et al. 2003). In terms of activation during hypoxia response HIF1α and HIF2α also act differently. They have different time courses, which means that HIF1α activation occurs at an earlier timepoint in hypoxic conditions (e.g. acute) than HIF2α and is also not so stable (Stiehl et al. 2012)(Holmquist-Mengelbier et al. 2006). Furthermore, despite the similar DNA-binding motif of HIF1α/β, both isoforms have different but overlapping binding sites in chromatin. However, they only transactivate partially those overlapping patterns of gene expression (Choudhry et al. 2014)(Hu et al. 2003)(Schödel et al. 2011).

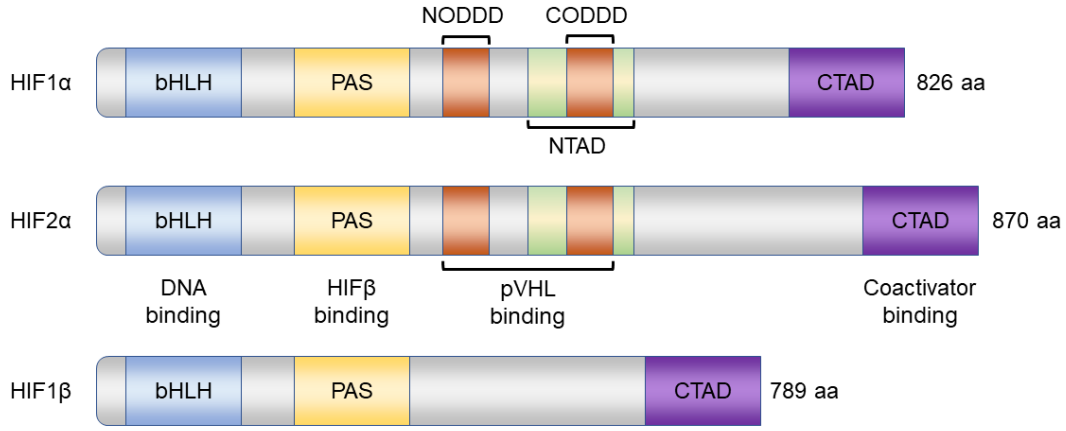

Figure 1: The structure of HIF1α, HIF2α and HIF1β. The basic helix-loop-helix (bHLH) structure as well as the Per-Arnt-Sim (PAS) domain are present in all subunits. The N- and C-terminal oxygen-dependent degradation domains (NODDD and CODDD) contain the proline residues which can be hydroxylated by the HIF-P4Hs. This hydroxylation leads to a binding site generation for the von Hippel-Lindau (VHL) tumor suppressor protein. In HIF1α and HIF2α there are also two transactivation domains (TAD), the N-terminal (NTAD) and C-terminal (CTAD) which are responsible for coactivator binding. Adapted and modified from (Chowdhury, Hardy, and Schofield 2008).

1.2.1.4 HIF1α and HIF2α target genes

The first known and most popular HIF1α target gene is human erythropoietin (EPO). HIF1α acts here as the key regulator by binding to the enhancer site and activating the transcription process for EPO (Wang and Semenza 1996). Since then many more target genes/functions of HIF have been identified. It has been shown that the stabilization of HIF1α and HIF2α results in differences in their gene regulation in response to hypoxia and disease states which could be observed for different forms of cancer (Keith, Johnson, and Simon 2012). For instance, in specific cells like endothelial cells there are 701 and 1454 genes known to be regulated by HIF1α and HIF2α, respectively. Other cell types even have up to 1807 DNA binding sites for HIF1α (HepG2 cells) and up to 3240 (HKC-8 cells) for HIF2α (Smythies et al. 2019). The higher number of activated genes and binding sites for HIF2α means that it regulates a more functionally and diverse set of target genes in comparison to HIF1α (Downes et al. 2018). In contrast, other cell types express HIF1α as the predominantly active form. This shows the highly diverse expression pattern of HIF isoforms in different tissues (Hu et al. 2006)(Mole et al. 2009). Interestingly the ratio of HIF1α and HIF2α for common binding sites in different cell types are very similar. Also, distinct patterns in terms of the binding distance to promoters could be shown in a recent study. HIF1α binds more closely to the transcriptional start sites whereas HIF2α binds more distantly (Smythies et al. 2019). However, both subunits have the ability to bind to the endogenous HREs within promoters of hypoxia related genes but are not able to activate genes which are specific for HIF1α or HIF2α (Hu et al. 2007). The complete number of known direct transcriptional target genes of HIF1α and HIF2α is estimated to be over 2000 (Downes et al. 2018, Semenza & Prabhakar 2018).

HIF directly regulates genes from many different areas among these are genes of the energy metabolism, growth and apoptosis, and angiogenic signaling (Fig. 2)(Schofield and Ratcliffe 2004). Both HIF1α and HIF2α have exclusive target genes as well as responsive genes to both isoforms (Holmquist-Mengelbier et al. 2006)(Hu et al. 2003)(Raval et al. 2005a). For instance HIF1α target genes contribute to processes in tumor metastasis, angiogenesis, energy metabolism, cell differentiation and apoptosis (Wenger, Stiehl, and Camenisch 2005)(Liao et al. 2009). Unique targets for HIF1α are the glycolytic enzymes phosphoglycerate kinase and lactate dehydrogenase-A, carbonic anhydrase-9 and the pro-apoptotic gene *BNIP-3* (Hu et al. 2003)(Sowter et al. 2001)(Raval et al. 2005a)(Grabmaier et al. 2004). Whereas genes which are exclusively regulated by HIF2α encode CYCLIN D1, Twist-related protein 1 (*TWIST1*) embryonic transcription factor OCT-4, transforming growth factor α (*TGF-α*) and the red blood

cell stimulating EPO (WARNECKE et al. 2004) (Baba et al. 2003)(Raval et al. 2005b)(Gunaratnam et al. 2003)(Covello et al. 2006)(Gort et al. 2008)(Gruber et al. 2007). Though genes responsible for the vascular endothelial growth factor (*VEGF*), adrenomedullin and the glucose transporter 1 (*GLUT-1*) are regulated by both HIFα subunits (Wiesener et al. 1998)(Hu et al. 2003). But the extend and individual roles of HIF1α and HIF2α remain unclear for many tissues still until now. A recent study focusing on the analysis of endothelial cells could characterize the individual roles of each subunit. HIF1α regulated primarily metabolic related genes whereas HIF2α had an impact on regulating angiogenic extracellular signaling, guidance cues and extracellular remodeling factors (Downes et al. 2018).

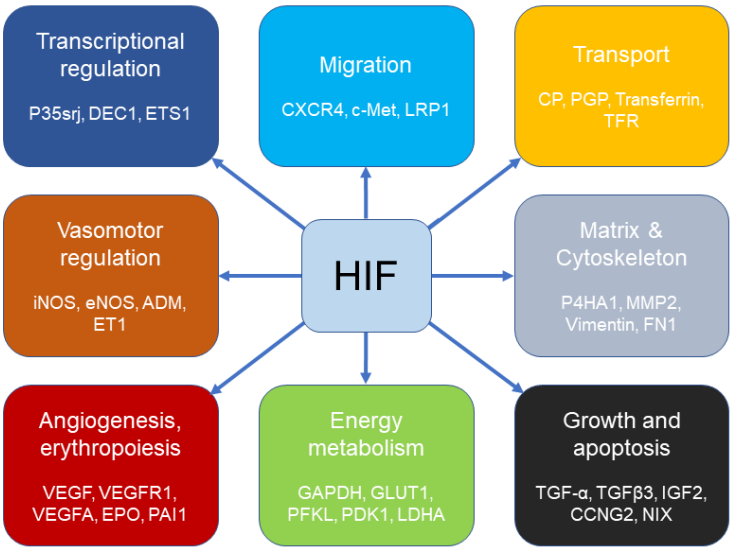

Figure 2: Representative HIF target genes. The current number of direct HIF target genes is estimated to exceed 2000. HIF1α and HIF2α target genes are not separated in this figure. Only examples of the most important functional categories are shown. Abbreviations and explanations: p35srj is a 35-kDa protein that contains a serine/glycine-rich junction; DEC1: differentiated embryo chondrocyte; ETS1: DNA-binding domain that defines a family of transcription factors; CXCR4: chemokine receptor 4; c-Met: tyrosine-protein kinase Met; LRP1: LDL receptor related protein 1; CP: ceruloplasmin; PGP: multidrug resistance P-glycoprotein; TFR: transferrin receptor; P4HA1: collagen propyl 4-hydroxylase alpha I; MMP2: matrix metalloproteinase 2; FN1: fibronectin 1; TGFα: transforming growth factor alpha; TGFβ3: transforming growth factor beta 3; IGF2: insulin like growth factor 2; CCNG2: Cbp/p300-interacting transactivator; NIX: BCL2 interacting protein; GAPDH: glyceraldehyde-3-phosphate; GLUT1: glucose transporter 1; PFKL: phosphofructokinase L; PDK1: pyruvate dehydrogenase kinase 1; LDHA: lactate dehydrogenase A; VEGF: vascular endothelial growth factor; VEGFR1: VEGF receptor 1; VEGFA: vascular endothelial growth factor A; EPO: erythropoietin; PAI1: plasminogen activator inhibitor 1; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase; ADM: adrenomedullin; ET1: endothelin 1. Figure adapted and modified from (Schofield and Ratcliffe 2004)

1.2.1.5 Regulation of HIF

Under normoxic conditions the HIFα subunits are very unstable and are degraded very fast, within 5 minutes (Huang et al. 1996). The regulation of HIFα takes place on a post-translational level via HIF-p4Hs and is the most common form of HIFα regulation. However, transcriptional and translational regulation of HIFα by kinases has also been shown to be important regulatory mechanisms but needs further investigation (Kietzmann, Mennerich, and Dimova 2016).

Under normoxic conditions the proline residues Pro402 and/or Pro564 of HIF1α or Pro405 and/or Pro531 of HIF2α which are located in the ODDD become hydroxylated by HIF-P4Hs (Kaelin 2005)(Kaelin and Ratcliffe 2008)(Chowdhury, Hardy, and Schofield 2008). This reaction requires besides oxygen (O_2) also co-factors like iron (Fe^{2+}), 2-oxoglutarate (2-OG) and ascorbate (Kaelin and Ratcliffe 2008). The hydroxylation of the proline residues leads to a binding site generation for the von Hippel-Lindau (VHL) tumor suppressor protein. VHL binds to HIFα and interacts with the protein Elongin C, thus recruiting an E3 ubiquitin-protein ligase complex which is polyubiquitinating HIFα. This state of polyubiquitination targets it for the degradation by the 26S proteasome (Stebbins et al. 1999)(Bruick and McKnight 2001)(Epstein et al. 2001)(Berra et al. 2003).

In hypoxia this degradation process does not happen. The oxygen dependent HIF-P4Hs cannot hydroxylate the proline residues and a stabilization of HIFα takes place. Stabilized HIFα translocates with help of a nuclear localization sequence which allows binding to importin α/β complexes from the cytosol to the nucleus. In the nucleus, as previous mentioned, the HIFα subunit forms a dimer with the HIFβ subunit and the transcriptional co-activator CBP/p300 binds to the CTAD of HIFα. This enables the transcription initiation complex (TIC) to bind the HREs within promoters of hypoxia related genes and initiate their transcription (Fig. 3) (Kaelin and Ratcliffe 2008)(Depping et al. 2008).

Another HIF regulating mechanism is led by factor inhibiting HIF1α (FIH1). FIH1 also belongs as well as HIF-P4Hs to the non-haem, Fe^{2+}-dependent, 2-OG-dependent oxygenase superfamily. At sufficient oxygen concentrations it hydroxylates the asparagine residue 803 in HIF1α and 851 in HIF2α in the CTAD domain. This blocks the binding of the transcriptional co-activators CBP and p300 and thereby suppressing its transcriptional activity (Kaelin 2005)(Schofield and Ratcliffe 2004)(Hewitson et al. 2002). It has been shown that FIH is not as sensitive as HIF-P4Hs to low oxygen concentrations. This means it can still hydroxylate proline residues when HIF-P4Hs have already stopped working. Both subunits also show

different sensitivities to FIH mediated inactivation as HIF1α appears more sensitive than HIF2α (Koivunen et al. 2004)(Kaelin and Ratcliffe 2008).

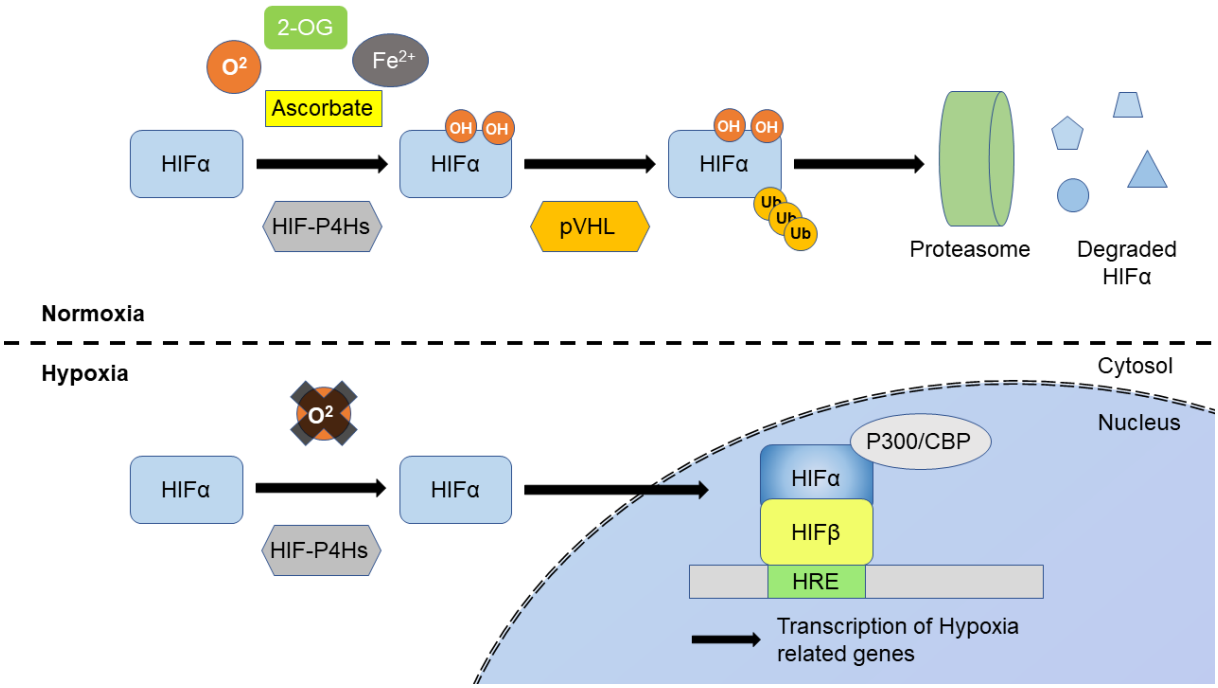

Figure 3: Schematic representation of the hypoxia response pathway. In normoxia HIF-P4Hs hydroxylate the HIFα subunit and targets it for the binding of von Hippel-Lindau protein (pVHL). Binding of pVHL leads to polyubiquitination followed by proteasomal degradation of HIFα. Hydroxylation of HIFα by HIF-P4Hs requires molecular oxygen (O_2), iron (Fe^{2+}), ascorbate and 2-oxlutarate (2-OG). Under hypoxic conditions, the HIF-P4Hs are not able to hydroxylate HIFα due to the absence of O_2 and it escapes proteasomal degradation. It translocates to the nucleus and forms a dimer with HIFβ. The transcription co-activator P300/CBP binds to the dimer and the complex binds to the hypoxia-response element (HRE) and leads to activation of HIF-target genes.

1.2.1.6 HIF prolyl 4-hydroxylases

In 2001 a novel prolyl 4-hydroxylase (P4H) family was discovered by two independent groups which appeared to be the master regulator of hypoxia response (Epstein et al. 2001)(Bruick and McKnight 2001)(J. Myllyharju 2013). The members of this prolyl 4-hydroxylase family were called HIF-P4Hs due to their active role in controlling HIF and thus the hypoxia response pathway. HIF-P4Hs belong to the Fe^{2+}-dependent, 2-OG-dependent oxygenase superfamily and like the name already indicates those enzymes require Fe^{2+}, 2-OG and O_2 for their catalytic function. Simpler animals like *Drosophila melanogaster* and *Caenorhabditis elegans* were found to have only one HIF-P4H gene while there are three known isoenzymes in vertebrates, HIF-P4H-1, HIF-P4H-2 and HIF-P4H-3, from which HIF-P4H-2 is the most abundant one and also the major regulator of HIFα stability (Ivan et al. 2002)(Centanin, Ratcliffe, and Wappner 2005)(Bruick and McKnight 2001)(Epstein et al. 2001). The cellular localization of HIF-P4Hs 1-3 vary from each other. HIF-P4H-1 is located exclusively in the nucleus whereas HIF-P4H-

2 is mainly located in the cytoplasm and HIF-P4H-3 is homogeneously distributed in both (Metzen et al. 2003)

HIF-P4Hs mRNA expression can be found in many cell lines and vertebrate tissues. Considering their expression levels there is a strong difference between the three isoenzymes, tissue wise. HIF-P4H-1 is the only isoform which can be found in the testis and has its highest expression in the placenta, while HIF-P4H-2 is relatively uniformly expressed in all tissues studied. The highest expression of HIF-P4H-3 can be observed in cardiac tissue and all three isoenzymes are expressed in the kidney (Hirsilä et al. 2003)(Lieb et al. 2002)(Cioffi et al. 2003)(Willam et al. 2006)(Appelhoffl et al. 2004).

There is also an additional enzyme, a transmembrane P4H (P4H-TM). It is located in the endoplasmic reticulum (ER) membrane with its active site within the ER. Like the other HIF-P4Hs it can hydroxylate HIFα *in vitro* and its knockdown by siRNA in cultured cells had an influence on HIFα levels (Koivunen, Tiainen, et al. 2007)(Oehme et al. 2002). The expression pattern of P4H-TM is more limited - the highest mRNA levels can be detected in the brain of humans and zebra fish (Koivunen, Tiainen, et al. 2007)(Hyvärinen et al. 2010)

1.2.1.7 Structure and reaction mechanism of HIF-P4Hs

Human HIF-P4Hs 1-3 vary in size from each other. HIF-P4H-1 is 407 aa, HIF-P4H-2 426 aa and HIF-P4H-3 is composed by 239 aa. However they share 42- 59% sequence identity (Fig. 4) (Bruick and McKnight 2001)(Epstein et al. 2001)(Ivan et al. 2002). The P4H-TM polypeptide has 502 residues and a transmembrane domain between residues 59 and 82 (Koivunen, Tiainen, et al. 2007)(Oehme et al. 2002). Crystallization studies of HIF-P4H-2 could show that its catalytic domain contains a double-stranded β-helix jelly-roll fold in the Fe^{2+}-binding motif which can be found in any 2OG-dependent dioxygenases (McDonough et al. 2006)(Chowdhury et al. 2009)(Illingworth et al. 2010).

This C-terminal catalytic domain of HIF-P4H-2 at position 294-392 aa possesses conserved residues for Fe^{2+} binding: H313, D315 and H374. For binding 2OG, there is an arginine at position 383 (McDonough et al. 2006)(Choi et al. 2005). The N-terminal domain in HIF-P4H-2 contains a motif which is essential for nuclear export, it lays in between aa 6 and 20 (Pientka et al. 2012).

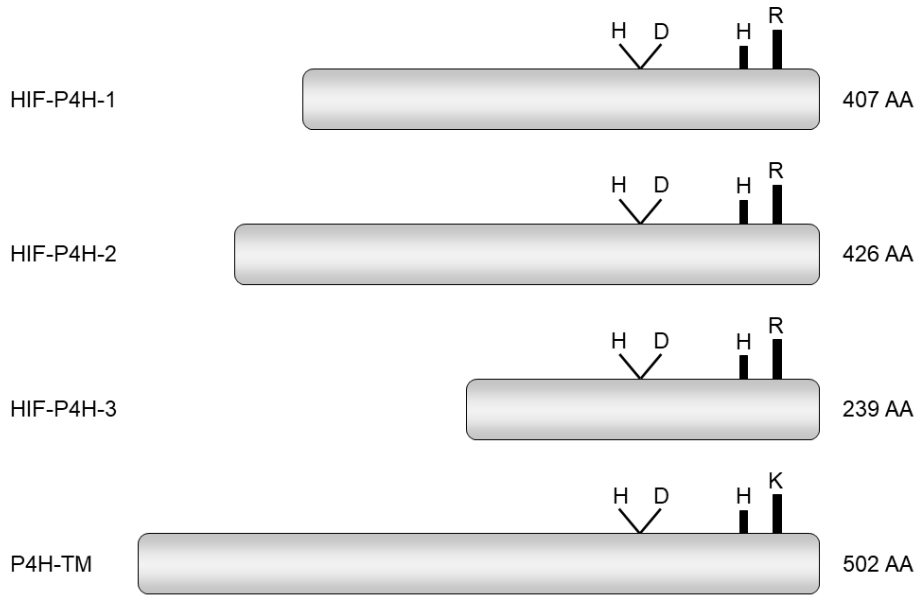

Figure 4. Schematic structure of the HIF-P4H isoenzymes. Catalytically critical residues which are able to bind the Fe^{2+} atom (H: histidine, D: aspartate) and the C-5 carboxyl group of 2-oxoglutarte (R: arginine) are shown above the polypeptides. The P4H-TM differs from the other isoenzymes by its C-5 carboxyl binding residue of 2-oxoglutarate being a lysine (K) instead of histidine. The number of amino acids (aa) of each isoenzyme are shown on the right. Figure adapted and modified from (Johanna Myllyharju 2008).

The reaction of HIF-P4Hs is highly dependent on oxygen. Due to its K_m values for O_2 which are slightly above the atmospheric concentration it is indicated that HIF-P4Hs are very sensitive oxygen sensors (Hirsilä et al. 2003). For the hydroxylation of HIFα, HIF-P4Hs requires O_2, 2OG, Fe^{2+} and ascorbate which is not used in the reaction. It acts as an alternative oxygen acceptor for uncoupled reaction cycles (Kaelin and Ratcliffe 2008)(Johanna Myllyharju 2008)(Johanna Myllyharju 2009). Those cofactors bind in a specific order to the catalytic site of HIF-P4Hs to which Fe^{2+} enters first followed by 2OG, the substrate and last O_2 (Schofield and Ratcliffe 2004)(Loenarz and Schofield 2011). In this reaction one oxygen atom is used for the hydroxyproline residue of HIFα and the other is involved in the oxidative decarboxylation of 2-OG(Kaelin and Ratcliffe 2008). Not only oxygen is a limiting factor for the HIF-P4Hs. Their catalytic activity also depend on the cellular availability of the mentioned co-factors (J. Myllyharju 2013). There are some studies which indicate that reactive oxygen species (ROS) are increasingly produced by mitochondria under hypoxic conditions which are thought also to limit/inhibit the activity of HIF-P4Hs (Nakazawa, Keith, and Simon 2016)(Lee et al. 2016). In addition, structural analogues of 2OG and other metabolites such as fumarate, malate, oxaloacetate, pyruvate, citrate, isocitrate and succinate can bind to HIF-P4Hs and inhibit their activity (Isaacs et al. 2005)(Koivunen, Hirsilä, et al. 2007)(Lu et al. 2005)(Selak et al. 2005)(Koivunen et al. 2012)

1.2.2 Pregnancy

1.2.2.1 Maternal lipid, glucose and energy metabolism during pregnancy

The concept of metabolism describes chemical changes which are continually occurring in living cells and its discovery can be dated back to the 18th century. The process itself can be divided into two processes which have opposing effects, anabolism and catabolism. Anabolism is the process in which food or nutritive matter is build up into living material, in contrast to catabolism where the protoplasm is degraded into more incomplex material.

Maternal metabolism represents a special kind of metabolism, because of its differences to the "normal" non-pregnant metabolism. It was first interpreted as the introduction of a parasite to the normal metabolism which was then flawed later for a couple of reasons (David R. Hadden and McLaughlin 2009). Maternal metabolism is adapted in order to optimize the outcome and growth of the fetus and changes significantly during the process of pregnancy. Early stages of pregnancy until the end of the second trimester can be characterized by an anabolic phase during which the mother increases fat stores by an enhanced *de novo* lipogenesis. Those stored nutrients are to meet the feto-placental and maternal demands of the catabolic phase in the last trimesters and later on lactation (Lain and Catalano 2007)(Zeng, Liu, and Li 2017a).

The first state in pregnant women is maternal hyperphagia, a state of compulsive overeating over a prolonged period of time which is progressively increasing. Through this process, the availability of exogenous metabolic substrates for the developing fetus is growing (Murphy and Abrams 1993). This comes along with the previously mentioned *de novo* lipogenesis. The increased *de novo* lipogenesis is due to a decreased adipose tissue lipolytic activity combined with an augmented capacity of maternal tissues to provide intracellular glycerol and glucose, which can be explained by an increase of the lipoprotein lipase (LPL) in adipose tissue (Herrera and Ortega-Senovilla 2014). This enzyme is responsible for the hydrolysis of lipoproteins and triglycerides. Everything together leads to a net accumulation of triglycerides (Thenen and Mayer 1975)(Zeng, Liu, and Li 2017b).

The higher levels of available glucose for the lipogenesis can be explained due to an essential change of glucose metabolism. In early pregnancy a decrease of fasting glucose values can be observed which goes along with a progressive rise in postprandial glucose levels in later pregnancy. The lower fasting glucose values are accompanied by a rise of plasma free fatty acids and enhanced ketogenesis as well as a decrease in plasma amino acids mainly due to an increase of the plasma volume in early pregnancy (B E Metzger n.d.). Increasing postprandial

glucose levels can be observed for the first time during the second trimester. These higher glucose levels are due to developing maternal insulin resistance which directs the energy usage more to the fetus than to the maternal tissues to cover its needs during growth and development (Lain and Catalano 2007).

In the third trimester the metabolism switches to a net catabolic state. In this phase there is an enhanced breakdown of fat deposits from maternal tissues as a consequence of increased adipose tissue lipolytic activity. This is driven by increased expression and activity of hormone sensitive lipase in the white adipose tissue (Martineau et al. 2015) as well as decreased levels and activity of LPL (Pujol et al. 2005). The liver plays an important role in that phase by converting non-esterified fatty acids (NEFA) and glycerol into acyl-CoA and glycerol-3-phopshate, respectively and by partial re-esterification for synthesis of liver triglycerides. Those triglycerides are then transferred to native very low-density lipoproteins (VLDL) particles and released into the maternal circulation. NEFA and glycerol can also be used for energy production and ketone body synthesis by oxidizing NEFA to acetyl-CoA and using glycerol for glucose synthesis (Zeng, Liu, and Li 2017a).

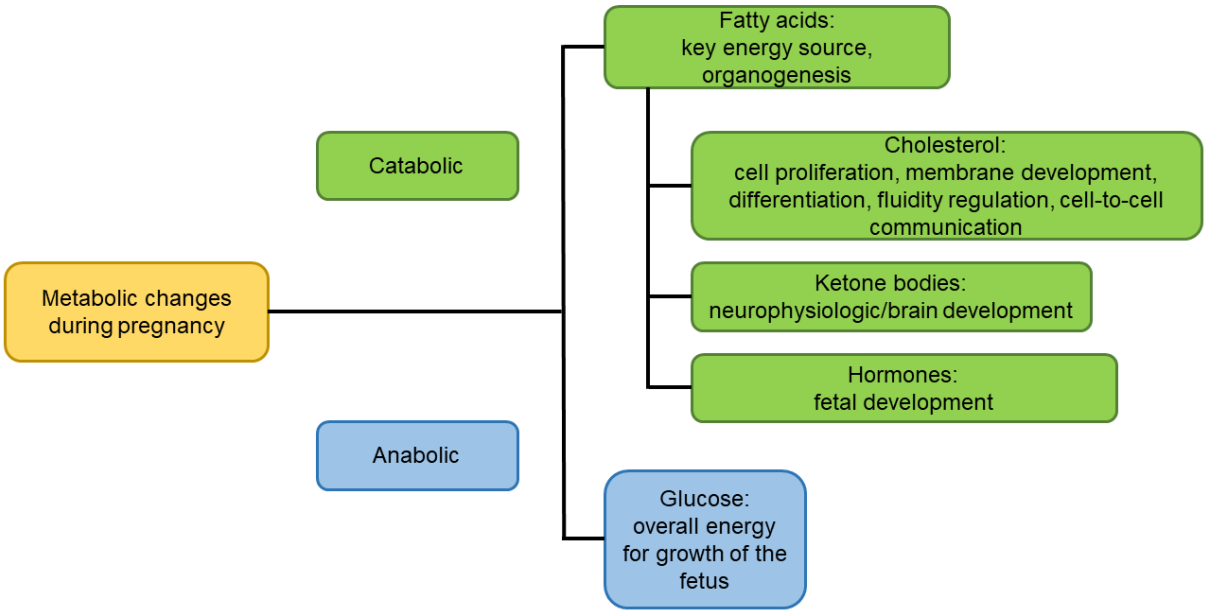

Figure 5: Overview of metabolic changes during pregnancy. Figure is showing the anabolic and catabolic changes in the metabolism of a pregnant women. Figure adapted and modified from (Zeng, Liu, and Li 2017b).

1.2.2.2 Pregnancy at high altitude

Worldwide an estimated number of 140 million people live permanently at altitudes higher than 2500 m above sea-level which is considered as high altitude due to the measurable fall of arterial O_2 saturation. Those people are the greatest group affected by O_2 restriction during pregnancy (E. Krampl 2002). High altitude conditions during gestation have a strong impact on the birthweight of a newborn. It is associated with a lower than average birthweight among different populations (G. M. Jensen and Moore 1997)(Levine et al. 2015)(Mortola et al. 2000). This effect is reduced in populations with a long ancestry living at high altitudes such as the Tibetans and Andeans (L.G., S.M., and C.G. 2011)(Soria et al. 2013). A study reported that for every 1000 m above sea level the birth weight reduces by around 100 g (Soria et al. 2013). This effect is comparable with moderate smoking (G. M. Jensen and Moore 1997) and also increases the incidence for intrauterine growth restriction by nearly three-fold (G. M. Jensen and Moore 1997)(Lichty et al. 1957)(Unger et al. 1988)(Julian et al. 2007). Intrauterine growth restriction causes many different pathologies like preeclampsia, a complication characterized by high blood pressure and signs of damage to other tissue and gestational hypertension (Keyes et al. 2003). There are also some physiological changes which come along with pregnancy at high altitude like lower fetal heart rate, higher uterine blood flow and dilated placental vessels. Those are all adaptations to compensate for lower oxygenation of the fetus (Ritchie et al. 2017).

Pregnant woman at high altitudes are facing the problem to sustain aerobic metabolism due to the reduced availability of oxygen (E. Krampl 2002). It affects next to the reduction in birth weight also the duration of the pregnancy. In terms of birth weight it is the second most important and in duration of pregnancy the most important factor affecting these conditions (G. M. Jensen and Moore 1997) (Julian et al. 2007)(E. Krampl 2002).

In terms of glucose metabolism during pregnancy, high altitude also renders the availability of energy for the fetus. Like described before, women develop an insulin resistance in the second half of the pregnancy in order to make more glucose available for the growing needs of energy of the fetus. Pregnant women at high altitudes show an opposing effect as their fasting glucose values are much lower compared to the ones living at lower levels; thus, the energy source for the fetus is more limited. Those women showed as well lower insulin plasma concentrations and decreased insulin sensitivity (Elisabeth Krampl et al. 2001). Therefore, it is very likely that the observable increase of glucose in maternal tissue is due to an insulin - independent increase in glucose utilization (E. Krampl 2002).

1.2.2.3 Hypoxia and pregnancy

Hypoxia is crucial for fetal development and a persistent status in all vertebrates. It drives vasculogenesis/angiogenesis, hematopoiesis and chondrogenesis (Giaccia, Simon, and Johnson 2004). For comparison normal adult arterial Po_2 is between 80-100 mmHg while the highest Po_2 found during late gestation in the umbilical vein ranges between 22-32 mmHg (Iwamoto et al. 1989)(Soothill et al. 1986). There are even transient periods where the fetal vascular Po_2 decreases for 6-8 min by 10-25 % during spontaneous uterine contractions (Jansen et al. 1979).

In contrast to the physiological hypoxia during gestation, non-physiological hypoxia with restricted oxygen supply for the fetus can have lasting negative consequences. Complications caused by the non-physiological fetal hypoxia are among the top 10 causes of fetal death (Anderson 2002). It can be caused by the previously mentioned living at high altitudes, hypertension, anemia, pulmonary disease, preeclampsia and through actively induced factors like drug abuse and smoking (G. M. Jensen and Moore 1997). Prenatal hypoxia is associated with many pathologies in later life. There are evolved fetal defense mechanisms to which redistribution of blood flow towards essential vascular beds like the brain, away from peripheral circulations for short-term hypoxia belongs (D. A. Giussani et al. 1993). The redistribution of blood flow persists when the duration of hypoxia is prolonged. Although this persistent blood flow towards essential tissues ensures fetal survival, it comes along with several side-effects. It causes asymmetric fetal growth restriction (Morrison 2008) as well as ventricular and aortic wall thickening in rats and chickens (Salinas et al. 2010)(Camm et al. 2010)(Rouwet et al. 2002). The effect of blood flow redistribution cannot compensate for more severe hypoxia in early development and can cause opposing effects, switching the cardiac phenotype to myocardial thinning (Tintu et al. 2009). Intrauterine hypoxia is not only a problem for the developing fetus but it also affects the development later in life and can cause heart and other cardiovascular diseases (Dino A. Giussani et al. 2012).

1.2.2.4 Gestational diabetes mellitus

Hyperglycemia which develops during gestation and resolves after birth has been recognized for over 60 years (D. R. Hadden 2008). The term gestational diabetes was firstly used in 1957 by the physician Elsie R. Carrington (Carrington, Shuman, and Reardon 1957). However, the first description of a diabetic pregnant woman with a big baby was in 1824 in the Charité Hospital, Berlin (De diabete mellito graviditatis symptomate - Heinrich G. Bennewitz - Google Books n.d.)

GDM has a very broad definition to which any degree of hyperglycemia firstly recognized during pregnancy belong. This can range from mild impaired glucose tolerance or impaired fasting glucose to glucose levels which are common in diabetic patients. More severe cases of hyperglycemia during pregnancy were uncommon earlier but are becoming more and more prominent due to the rise of diabetic and obese people as well as the trend towards childbearing at advanced age (Menke, Casagrande, and Cowie 2018). There is still no globally accepted diagnostic protocol for GDM. However there are criteria defined by the International Association of Diabetes in Pregnancy Study Groups (IADPSG) and confirmed by the WHO which are distinguishing between women who would be diagnosed with diabetes even without pregnancy and women which develop it during pregnancy (Boyd E. Metzger 2010)(WHO | Diagnostic criteria and classification of hyperglycaemia first detected in pregnancy 2013)(Omori and Jovanovic 2005).

It is very difficult to define the prevalence of GDM worldwide because the percentage of cases ranges from 6.1 to more than 30 %. Due to the lack of uniformly diagnostic criteria and screening standards it is very difficult to compare the prevalence of GDM between different countries. However, studies which refer to previously mentioned criteria indicate a global prevalence of GDM (Fig 6) (McIntyre et al. 2019)(Zhu and Zhang 2016). The highest prevalence of GDM can be found in the Middle East with a median of 15.2 %. Furthermore, South East Asia (median 15.0 %) and Western Pacific (median 10.3%) show a higher GDM prevalence compared to Europe (median 6.1 %) which have lowest prevalence but widest variation between countries. The overall prevalence of GDM worldwide is relatively consistent except for countries with a rising number in cases. It is very likely that higher numbers of GDM are due to the rising numbers of people with hyperglycemia outside pregnancy and that the first diagnosis of diabetes is done during pregnancy (McIntyre et al. 2019).

There are many identified risk factors for GDM like a history of gestational diabetes and family history of type 2 diabetes as well as advanced maternal age and ethnicity. Overweight or obesity, genetic factors, cigarette smoking or a unhealthy diet are as well on this list (McIntyre et al. 2019).

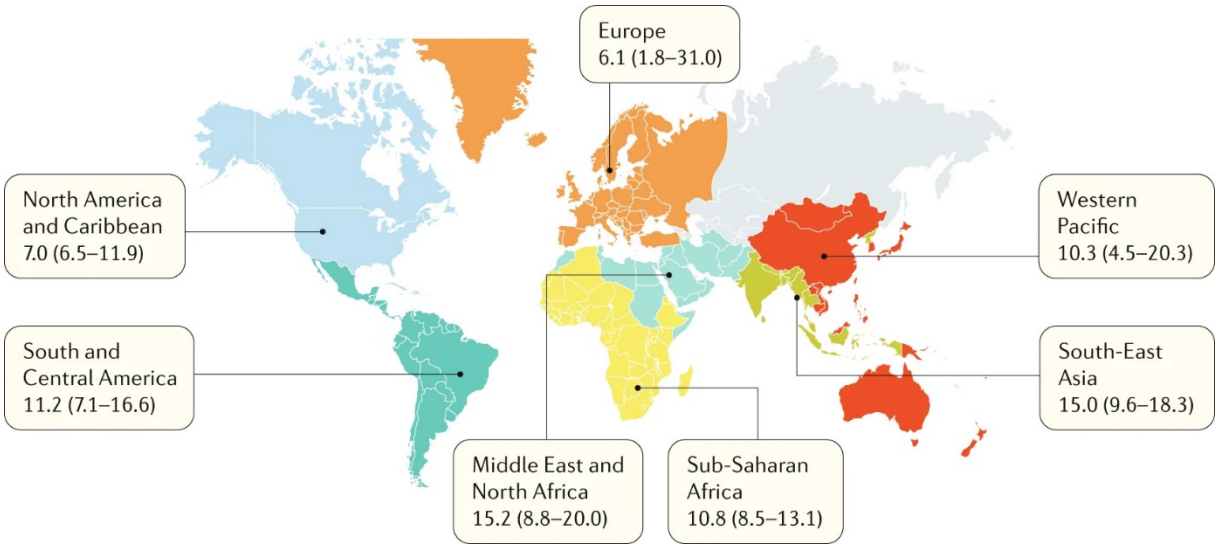

Figure 6: Median prevalence (%) of GDM by WHO between 2005-2018. Figure from (McIntyre et al. 2019).

For understanding the pathophysiological mechanisms in GDM it is important to consider the metabolic changes which occur during pregnancy. These changes include an increase in endogenous glucose production and a substantial increase in fasting insulin levels, like mentioned before. For women who develop GDM during late pregnancy there is evidence of a decreased insulin sensitivity before pregnancy (Catalano et al. 1999). Those women can still maintain normoglycemia during early pregnancy because of the ability of pancreatic β-cells to enhance their insulin production but in late pregnancy when insulin resistance is increased their response is inadequate, and results in hyperglycemia (Buchanan 2001). Also, the insulin signaling cascade which regulates the redistribution of glucose transporter type 4 (GLUT4) on the cell surface and therefore enables glucose uptake is impaired in women with GDM. As mentioned before there is a decreased insulin sensitivity in late pregnancy and this is further decreased in women with GDM (DeFronzo et al. 2015). In late pregnancy there are lower amounts of the signaling molecule insulin receptor substrate 1 (IRS1) in the skeletal muscle than in non-pregnant women. Also, the autophosphorylation of the insulin receptor β-subunit (IRβ) which activates a signaling cascade that is also responsible for the redistribution of GLUT4 on the cell surface is lower in women with GDM. Both factors result in a 25 % lower glucose uptake in skeletal muscle (Friedman et al. 1999). Also, inflammation which is liked to obesity can interfere with the insulin signaling cascade. Tumor necrosis factor (TNF) which is

commonly overexpressed in WAT of adipose people, can also decrease insulin sensitivity in pregnant women. It activates a specific signaling pathway which promotes the serine phosphorylation of IRS1 and thereby disturbs the insulin signaling cascade which also leads to decreased levels of insulin sensitivity (Hotamisligil et al. 1996)(Kirwan et al. 2004).

The main macronutrient which is capable of sustaining fetal growth is maternal glucose. In pregnant women with type 1 diabetes is observed that the pancreas of the fetus is reacting to prolonged hyperglycemia from early stages by accelerated maturation of pancreatic β-cells, which in turn results in hyperinsulinemia followed by hyperglycemia. The same mechanism is assumed to take place in women with GDM, but it stays undetected until diagnosis. Also, amino acids like arginine can stimulate the fetal pancreas and therefore cause hyperinsulinemia. Triglyceride and fat storage in white adipose tissue (WAT) is stimulated by fetal insulin as well, which leads to bigger amounts of fetal WAT (Eder et al. 2016).

Long-term epigenetic consequences of GDM caused by hyperglycemia are changes in DNA methylation and microRNA in fetal blood, skeletal muscle and adipose tissue. However, epipathogenetic consequences of those epigenetic changes for the fetus are not well-known yet (Houshmand-Oeregaard, Hjort, et al. 2017)(Houshmand-Oeregaard, Hansen, et al. 2017)(Hjort et al. 2018).

There are a couple of short- and long-term clinical consequences caused by GDM for the mother and child as well. Short-term consequences include operative delivery, fetal overgrowth, neonatal hypoglycemia, pre-eclampsia, polyhydramnios and birth canal lacerations (Pettitt et al. 1980)(Beischer et al. 1996)(Casey et al. 1997)(Aberg et al. 1997)(Persson and Hanson 1998)(Dorte Møller Jensen et al. 2003). With rising maternal glucose levels a graduated increase in the risk of maternal, fetal and neonatal complications can also be observed (Sermer et al. 1995)(Sacks et al. 1995)(Moses and Calvert 1995)(Dorte M. Jensen et al. 2001). Long-term consequences include a more than seven-fold increased risk of getting type 2 diabetes compared with normoglycemic pregnancies as well as a more than two times increased risk of getting pre-diabetes (Bellamy et al. 2009)(Hyvärinen et al. 2010)(Patel et al. 2016). Consequences of GDM for the offspring could be demonstrated in animal models where they simulated GDM. Those animals had increased risk of hyperglycemia, diabetes, obesity and cardiovascular disease (Harder et al. 2001)(Aerts and Van Assche 2006). These observations are very similar to the ones made in several clinical studies. In a study from Denmark 21 % of the offspring from women with GDM developed pre-diabetes or even diabetes which is an eight

times higher risk compared to the normal population. Also, the risk of getting metabolic syndrome and overweight was higher by four-fold and two-fold, respectively. The insulin sensitivity and the secretion were reduced as well (Tine Dalsgaard Clausen et al. 2008)(Tine D. Clausen et al. 2009). Another study with nearly 100000 participants could also show increased fasting glucose levels, insulin resistance, cardiovascular risk and adiposity (Grunnet et al. 2017).

2 EXPERIMENTAL PART

2.1 Aim of the Project

Recent studies have shown a reduction in the birthweight of mouse pups by hypoxia exposure of the dam which is similar to the observations of pregnant women living in higher altitude < 2500m. This birthweight reduction is caused by altering the maternal glucose and lipid metabolism and leads to an upregulation of the insulin sensitivity as well as improved glucose tolerance. Those dams show also a reduction of gonadal white adipose tissue (WAT).

A health condition many women are getting through pregnancy, especially ones with an increased body mass index (BMI > 25) is gestational diabetes mellitus (GDM). Women with those conditions often show symptoms like patients with diabetes which includes insulin resistance and an increased amount of serum lipids. Those conditions can lead to an increased birthweight and a variety of symptoms/health conditions in later life of the baby e.g. the metabolic syndrome. Because of the observed opposing effects of hypoxia and GDM during pregnancy, we investigated whether hypoxia will ameliorate the GDM symptoms in pregnant mice with insulin resistance.

2.2 Materials and Methods

2.2.1 Animals and hypoxic intervention

2.2.1.1 Animals and Diet Setup

The animal experiments were performed according to the Finnish Act on Animal experimentation (62/2006) and approved by National Animal Experiment Board of Finland. Female C57BL/6N mice between 4-5 months old have been used. Insulin resistance and obesity have been induced by feeding them *ad libitum* with high-fat diet (Special Diet Services, #820350, 38 % carbohydrates, 20 % animal lard, 23 % protein, 28.43 kJ/g) and sweetened condensed milk (Dovgan, 56.4 % sugar, 8 % fat, 6.9 % protein, 13.72 kJ/g) for 6-8 weeks before and throughout pregnancy. Control-fed female mice received standard chow (Teklad Global Rodent diet T.2018C.12, 44.2 % carbohydrates, 6.2 % fat, 18.6 % protein, 13 kJ/g) throughout the experiment. Mice were mated overnight in normoxic conditions (21 % oxygen) and the presence of a vaginal plug was used to mark gestational/ embryonic day 0.5. Pregnant mice (E0.5) from the control and diabetic group have been assigned to normoxia and hypoxia (15 % O_2) by matching their insulin resistance (HOMA-IR) scores and body weight, respectively. The hypoxia groups went into a hypoxic chamber (Hypoxic Glove Box, Coy Laboratory Products, USA) with 15 % normobaric oxygen concentration and the other group was kept in the same room under normoxic conditions. This setup created 4 groups of pregnant mice: normal chow-fed under normoxia, normal chow-fed under hypoxia, high-fat diet-fed under normoxia and high-fat diet-fed under hypoxia. Weight of the dams was taken 5 days per week. Pregnant mice were sacrificed at E9.5 and E17.5, and blood and terminal tissue samples were collected.

2.2.1.2 Glucose Tolerance Test (GTT) and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR)

The GTT was performed on pregnant dams (E17.5) after 4 h fasting. During the GTT the mice were anesthetized with fentanyl/fluanisone and midazolam (0,004 ml / g body weight) injected subcutaneously. Glucose-solution (2 mg/g body weight) was injected intraperitoneally and the blood glucose concentrations were measured directly from the hind limb at baseline (0 min) and 15, 30, 60 and 120 min after injection with a glucometer (Contour, Bayer). The homeostatic model assessment-insulin resistance (HOMA-IR) index was calculated from the fasted (baseline) blood glucose and serum insulin values using the formula: ((fasting insulin (pmol/l) × fasting glucose (mmol/l))/156.65).

2.2.2 Blood Analysis

2.2.2.1 Blood Hemoglobin Analysis

Baseline blood hemoglobin levels were measured spectrophotometrically directly from the hind limb of anesthetized mice before GTT with HemoCue Hb 201 analyzer.

2.2.2.2 Blood Lactate Analysis

Baseline blood lactate was measured by using enzymatic-amperometric detection (Lactate Scout + -meter; EKF Diagnostics) directly from the hind limb of anesthetized mice before the GTT.

2.2.3 Terminal Serum Analysis

2.2.3.1 Serum Extraction

The terminal blood from the dams was collected at sacrifice. Serum was obtained by allowing the blood samples to stand at room temperature for 1-2 h and subsequent centrifugation at 3000 *g* for 20 min at 4 ºC (Eppendorf centrifuge 5417C). The upper phase (serum phase) was collected and stored at -80 °C.

2.2.3.2 Total Cholesterol

For determination of total serum cholesterol an enzymatic kit was used (CHOL2, #04718917190, Lot. Nr. 35783101, Roche Diagnostics). The principle of the assay is a homogeneous enzymatic colorimetric test. Cholesterol esters are broken down by cholesterol esterase into free cholesterol and fatty acids. The oxidation of free cholesterol towards Δ^4-cholestenone and H_2O_2 is catalyzed by cholesterol oxidase. H_2O_2 is then forming with 4-aminoantipyrin and phenol under the catalyzing effect of peroxidase a red quinone imine dye. The intensity of the dye is directly proportional to the concentration of the cholesterol.

Table 1: Reaction mix serum cholesterol analysis.

Serum	2µl
Reagent	47 µl
dH_2O	93 µl
Total	142 µl

The reaction mix was incubated for 10 min at room temperature on a shaker (Titramax 1000, Heidolph) at 250 rpm and the absorbance at 512/659 nm was measured with Infinite M1000 Pro Multimode Plate Reader (Tecan).

- ➔ Standard: Duocal Multi (Lyophilized Calibration Serum for Clinical Chemistry), #105300, Lot. Nr.:18339
- ➔ Control: Seronorm Lipid, #100205, Lot. Nr. 1611618

2.2.3.3 HDL-Cholesterol

Serum HDL-Cholesterol concentration was measured with an enzymatic kit (HDLC4, #07528604190, Lot. Nr. 35735501, Roche Diagnostics). The principle of the assay is a homogeneous enzymatic colorimetric test. Non-HDL lipoproteins and chylomicrons are combined with polyanions and a detergent forming a water-soluble complex. The enzymatic reaction of cholesterol esterase (CHER) and cholesterol oxidase (CHOD) towards non-HDL lipoproteins is prevented. Only HDL-particles can react with CHER and CHOD which defines enzymatically the concentration of HDL-cholesterol. CHER breaks down cholesterol esters into free cholesterol and fatty acids. The free cholesterol is oxidized by CHOD to Δ^4-cholestenone and H_2O_2. The hydrogen peroxide reacts in with 4-amino-antipyrine and N-ethyl-N-(3-methylphenyl)-N'succinylethylenediamine (EMSE) to form a dye in the presence of peroxidase. The intensity of the dye is proportional to the cholesterol concentration.

Table 2: Reaction mix serum HDL- cholesterol analysis.

Serum	2 µl
Reagent 1	120 µl
Reagent 2	40 µl
Total	162 µl

The reaction mix was incubated for 20 min at room temperature on a shaker (Titramax 1000, Heidolph) at 250 rpm. Absorbance at 583/659 nm was measured in Infinite M1000 Pro Multimode Plate Reader (Tecan).

- ➔ Standard: C.f.a.s Lipids (Calibrator for automated systems), #12172623, Lot. Nr.: 15464301
- ➔ Control: Seronorm Lipid, #100205, Lot. Nr.: 1611618

2.2.3.4 Triglycerides

Concentration of serum triglycerides was determined by an enzymatic kit (TRIGL, #04657594190, Lot. Nr.: 35078101, Roche Diagnostics) The principle of the assay is an enzymatic colorimetric test. The triglycerides are completely hydrolyzed by a lipoprotein lipase to glycerol and fatty acids. This reaction is followed by phosphorylation and oxidation of glycerol to dihydroxyacetone phosphate and H_2O_2. A red dye is then formed in the reaction of H_2O_2 with 4-aminophenazone and 4-chlorophenol. The intensity of the red dye is directly proportional to the triglyceride concentration.

Table 3: Reaction mix serum Triglyceride analysis.

Serum	2 µl
Reagent	120 µl
dH_2O	28 µl
Total	150 µl

The reaction mix was incubated for 10 min at room temperature on a shaker (Titramax 1000, Heidolph) at 250 rpm. Absorbance at 512/659 nm was measured in Infinite M1000 Pro Multimode Plate Reader (Tecan).

- ➔ Standard: Duocal Multi (Lyophilized Calibration Serum for Clinical Chemistry), #105300, Lot. Nr.:18339
- ➔ Control: Seronorm Lipid, #100205, Lot. Nr. 1611618

2.2.3.5 Ketoacids

Serum ketoacids were measured with the Beta-Hydroxybutyrate assay Kit (#MAK041, Lot. Nr.: 3G13K06320, Sigma-Aldrich) according to the manufacturer's protocol. The concentration is determined by coupled enzymatic reaction; the resulting colorimetric product intensity is proportional to the β-hydroxybutyrate concentration and its absorbance can be measured at 450 nm.

2.2.3.6 Free Fatty Acids

The concentration of free fatty acids in the serum was determined with the colorimetric/fluorometric Free Fatty Acid Kit (#ab65341, Abcam). Fatty acids are converted into their CoA derivatives. Those derivatives are then subsequently oxidized with the

generation of color or fluorescence. Octanoate and longer fatty acids were then quantified at 570 nm.

2.2.3.7 Glucagon

Quantitative determination of serum glucagon was done with the Mouse Glucagon ELISA Kit (#81518, Crystal Chem). The glucagon ELISA kit is based on the sandwich enzyme immunoassay principle. The kit provides a 96-well plate which is coated with monoclonal anti-glucagon antibodies which are capturing glucagon from the samples. The samples contain HRP-labeled (Horseradish Peroxidase) monoclonal anti-glucagon antibodies as well. During incubation the glucagon in the sample forms a complex with both antibodies on the surface of the plate. Afterwards the excessive HRP-labeled antibodies are removed and the HRP enzyme activity is determined with a TMB (3,3′,5,5′-tetramethylbenzidine) substrate. Absorbance was measured at 450/630 nm in Infinite M1000 Pro Multimode Plate Reader (Tecan)

2.2.3.8 Insulin

Insulin concentration of serum samples was measured with an ELISA sandwich assay kit (Ultra-Sensitive Mouse Insulin ELISA Kit, #90080, Crystal Chem). In the first reaction mouse insulin of the serum sample is bound to guinea pig anti-insulin antibody coated on microplate wells. In the second reaction HRP-labeled anti-insulin antibody binds to the anti-insulin antibody/mouse insulin complex. By adding TMB substrate the HRP enzyme activity is determined by measuring the absorbance at 450/630 nm in Infinite M1000 Pro Multimode Plate Reader (Tecan).

2.2.4 Tissue Analysis

2.2.4.1 Quantitative real-time PCR (qPCR) analysis

2.2.4.1.1 RNA Extraction Liver, Muscle

Total RNA was extracted from liver and muscle tissues by using the method described by Chomczynski et al., (Chomczynski 1993) and further purified by E.Z.N.A Total RNA Kit I (#R6834, Lot. Nr. R68340105241835122395, OMEGA Bio-Tek). In brief, ~ 100 mg tissue was homogenized in 1 ml TriPure (#11667157001, Lot. Nr. 94014220, Roche Diagnostics) and two stainless steel beads (#69989, QIAGEN) with a tissue homogenizer (QIAGEN Tissue Lyser). Samples have been homogenized for 5-10 min (liver) or 20-30 min (muscle) util no tissue pieces were any more visible. For dissociation of nucleoprotein complexes samples were incubated

for 5 min at 15-25 ºC. 0.2 ml chloroform was added, and the tubes were mixed for 15 s, and incubated for 5-10 min at 15-25 ºC. To separate the three phases the samples have been centrifuged at 12000 *g* for 15 min at 2-8 ºC (Eppendorf centrifuge 5417C). The upper phase containing the RNA (aqueous phase) was transferred to a new tube and precipitated with 0.5 ml 2-Propanol (#20842.330, Lot. Nr.: 17D244010, VWR Chemicals). Tubes were mixed by inversion and incubated for 5-10 min at 15-25 ºC. Samples have been centrifuged at 12000 *g* for 10 min at 2-8 ºC and the supernatant discarded. The resultant pellet has been washed with 75 % EtOH, centrifuged at 7500 *g* for 5 min at 2-8 ºC and air dried for 30 min in a fume hood. The air-dried pellet was then dissolved in 100 µl pre-warmed DEPC-water and incubated for 15 min at 55-60 ºC in a heat block (Lab Line Multi Blok Heater, Thermo Scientific). The purification of the impure RNA was done by using the E.Z.N.A. Total RNA Kit I according to the manufacturer's instructions and with an additional DNase I Digestion step using the RNase-Free DNase I Set (#E1091, Lot. Nr.: E109106181831121955, OMEGA Bio-Tek). The concentration of the purified RNA was measured using NanoDrop 2000 Spectrometer (Thermo Scientific).

2.2.4.1.2 RNA Extraction WAT

Isolation of total RNA from visceral white adipose tissue (WAT) was performed using the E.Z.N.A Total RNA KIT II (#R6934, Lot. Nr. R693401101318011235 60, OMEGA Bio-Tek), which is designed for tissues rich in fat, following the Total RNA Kit II – animal tissue protocol. In brief, ~ 100 mg of tissue was homogenized in 1 ml RNA-Solv Reagent and 2 stainless steel beads (#69989, QIAGEN) with a tissue homogenizer (QIAGEN Tissue Lyser). Samples have been homogenized for 5-10 min until no tissue pieces were visible anymore. The concentration *of the purified RNA was measured using NanoDrop 2000 Spectrometer (Thermo Scientific).*

2.2.4.1.3 cDNA Synthesis

The generation of cDNA for qPCR was done with qScript cDNA Synthesis Kit (#95047, Lot. Nr.: 026644, Quantabio) using the previously generated total RNA as template and following the manufacturer's protocol. For one reaction 1 µg total RNA was used. cDNA synthesis was performed in T100 Thermal Cycler (Bio-Rad) and the reaction is shown in the table below.

Table 4: cDNA synthesis reaction

RNA (1 µg)	variable
Nuclease-free water	variable
qScript Reaction Mix (5X)	4.0 µl
qScript Reverse Transcriptase	1.0 µl
Total	20.0 µl

Recommended thermal cycler program is shown below.

Table 5: cDNA synthesis thermal cycler program

1 cycle	22 ºC	5 min
1 cycle	42 ºC	30 min
1 cycle	85 ºC	5 min
	4 ºC	∞

2.2.4.1.4 Quantitative real-time PCR analysis

Quantitative real-time PCR analysis was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) by using iTaq Universal SYBR Green Supermix (Bio-Rad). Reaction mix and thermal cycling protocol are presented in the tables below.

Table 6: qPCR Reaction mix

iTaq Universal SYBR Green Supermix (2x)	5 µl
Forward primer (3 µM)	1.0 µl
Reverse primer (3 µM)	1.0 µl
cDNA template	2.0 µl
Nuclease-free H_2O	1.0 µl
Total	10.0 µl

Table 7: qPCR Thermal Cycling program

1	1 cycle	95 ºC	3 min
2	1 cycle	95 ºC	15 sec
3	1 cycle	60 ºC	45 sec
4	repeat 2 and 3		39 more times
5	1 cycle	95 ºC	10 sec

All primer sets used in the qPCR analyses are listed in the table below.

Table 8: Primer sequences for qPCR

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
Actb	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
PfkL	TGCAGCCTACAATCTGCTCC	GTCAAGTGTGCGTAGTTCTGA
Irs2	GTAGTTCAGGTCGCCTCTGC	TTGGGACCACCACTCCTAAG
Pdk1	AGGATCAGAAACCGGCACAAT	GTGCTGGTTGAGTAGCATTCTAA
Ccl2	CCTGCTGTTCACAGTTGCC	ATTGGGATCATCTTGCTGGT
Glut1	TCAAACATGGAACCACCGCTA	AAGAGGCCGACAGAGAAGGAA
Glut2	TTCCAGTTCGGCTATGACATCG	CTGGTGTGACTGTAAGTGGGG
Glut4	ACACTGGTCCTAGCTGTATTCT	CCAGCCACGTTGCATTGTA

2.2.4.2 Glycogen Determination

The glycogen concentrations of liver, skeletal muscle and kidney tissue were analyzed with the Glycogen Assay Kit (# 700480, Cayman Chemical). The principle of the assay is an enzyme based fluorometric test. β-D-glucose is being formed by the hydroxylation of glycogen with amyloglucosidase. Glucose oxidase is then oxidizing β-D-glucose to form hydrogen peroxide. Hydrogen peroxide is reacting in the presence of horseradish peroxidase with 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) to form the fluorescent product resorufin in a 1:1 stoichiometry manner. The fluorescence can then be measured with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm (Infinite M1000 Pro Multimode Plate Reader (Tecan)). The assay was performed according to the manufacturer's protocol. Tissue samples between 60-150 mg were taken. Samples were diluted prior the assay (muscle and kidney samples 1:10 and liver samples 1:70).

2.2.4.3 Liver triglyceride determination

Extraction of hepatic lipids was done by overnight digestion in an ethanol KOH-solution at +55 ºC followed by centrifugation for 5 min at 10000 *g* (Eppendorf centrifuge 5417C). The lipids in the supernatant were precipitated on ice with $MgCl_2$ and centrifuged for 5 min at 10000 *g*. The triglyceride concentration was then measured by the colorimetric method Triglyceride GPO-PAP (#11488872 216, Lot. Nr.: 616434-01 Roche Diagnostics).

2.2.5 Histological analyses

2.2.5.1 Tissue processing and thin slice cutting:

Tissues were fixed in 10% formalin (#FF119, Lot. Nr.: 080119, Oy FF-Chemicals Ab)) after sacrifice for 24 h. The formalin was removed, the samples were washed for 1.5 h under running tap water and transferred into 70 % ethanol. The samples were processed in the automated tissue processor (Tissue-Tek VIP Jr, Sakura) and embedded in paraffin (Tissue-Tek TEC, Sakura). 5 µm sections were cut by using a microtome (MIKROM HM 355S, Thermo Scientific) and transferred on microscope slides (SUPERFROST PLUS, Thermo Scientific). The slides were allowed to dry overnight at room temperature. All tissue slides were examined under Leica DM LB2 microscope and photographed using Leica DFC 320 camera.

2.2.5.2 HE-Staining:

WAT and liver tissue samples were stained with hematoxylin-eosin (HE) (Fischer et al. 2008). Eosin (Reagena) and Harri´s Hematoxylin (Sigma Aldrich) were used. Samples were mounted with Pertex (Histolab) and cover slips (Menzel-Gläser).

2.2.5.3 Hepatic steatosis and lymphocyte quantification:

The degree of hepatic steatosis and lymphocyte infiltration were scored from HE-stained liver samples as 0-1 corresponds to “none”, 2 corresponds to “moderate” and 3-4 corresponds to “severe”.

2.2.6 Statistical analysis:

Student´s two-tailed test was used for determining statistical significance between two groups. Grubb´s test was used to determine statistically significant outliers and the Fisher´s exact test for histological comparisons. The calculation of the areas under the curve were done with the

summary measures method. Data is presented as means ± standard error of the mean (SEM). P values ≤ 0.05 were considered as statistically significant.

2.3 Results

2.3.1 The impact of maternal obesity and hypoxia in mid pregnancy

To study the effects of hypoxia on gestational diabetes mellitus (GDM) in mice, obesity was induced by feeding 4-months-old C57BL/6NCrl dams with an obesogenic diet (OD) for 7 weeks before and throughout pregnancy. Mice fed a normal chow (NC) served as control. Animals were bred following an established protocol and the presence of a vaginal plug was marked at day E 0.5 after mating and was considered as the beginning of pregnancy. The groups were housed either in normoxic conditions ($O_2 \approx 21$ %) or in normobaric hypoxia ($O_2 = 15$ %, this corresponds to oxygen tension at 2700 m altitude) during gestation; mice were sacrificed at E 9.5 (mid pregnancy).

2.3.2 OD decreased the insulin sensitivity under normoxia but not under hypoxia

To study whether OD had an effect on glucose metabolism in mid pregnant mice, it was first compared whether blood glucose, serum insulin levels and homeostatic model assessment-insulin resistance (HOMA-IR) values at baseline (BL) and mid pregnancy were different. The data showed that at E 9.5 blood glucose and HOMA-IR scores were increased in NC-fed mice housed under normoxia when compared with the BL. Further dams fed an OD under normoxia showed a significant increase in blood glucose, HOMA-IR scores and additionally serum insulin levels. Their levels were even higher than in mice fed a NC indicating a lower insulin sensitivity caused by OD. An increase in all parameters from BL to E 9.5 could also be observed in both OD groups. However significant differences can only be seen in blood glucose and HOMA-IR values (Table 9).

When NC-fed mice were housed under hypoxia it was observed that blood glucose, serum insulin levels and HOMA-IR values were higher at E 9.5 when compared with the NC-fed mice housed under normoxia. There was even significant difference in insulin ($P \leq 0.001$) and HOMA-IR ($P \leq 0.01$) values between the NC-fed mice housed under normoxia and hypoxia at E 9.5 which alters with previous observations for mid pregnancy (Määttä et al. 2018). Further, glucose and HOMA-IR in OD-fed mice at E 9.5 housed under hypoxia were lower when compared with OD-fed mice at E 9.5 housed under normoxia. When comparing NC-fed with OD-fed mice at E 9.5, glucose and serum insulin levels were increased by about ~ 2-fold and HOMA-IR by about 3-fold under normoxia while this increase was only by about 1.2-fold under hypoxia for the parameters glucose and HOMA-IR. Insulin levels where even lower in the OD-

fed mice under hypoxia. (Table 9). Altogether, these data suggest there was a mid-pregnancy-induced insulin resistance in all groups, which was further increased by OD under normoxia but not under hypoxia.

Table 9: ***Maternal glucose levels are upregulated, and glucose tolerance is lowered in OD mice.*** *Table 1 shows a comparison of baseline and E 9.5 blood glucose values, serum insulin levels and the HOMA-IR scores of blood samples taken after 4 h fasting. Data are means. Grubbs´s test was used for outliers. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Student's two-tailed t-test was used for comparison between 2 groups*

	Glucose (mmol/l)		Insulin (ng/ml)		HOMA-IR	
	BL	E 9.5	BL	E 9.5	BL	E 9.5
N/NC	7.15	12.3***	1.14	1.05	8.78	15.82
H/NC	6.65	15.71**	1.10	2.55***	14.10	37.50***
N/OD	7.24	22.04***	1.33	1.96	9.64	48.68***
H/OD	7.47	18.97***	1.57	2.07	11.36	45.37**

2.3.3 Effects of OD and hypoxia on maternal and embryo weights

Independent of the oxygen tension, mice fed an OD were heavier than NC-fed mice before and during the pregnancy until E 9.5 (Fig. 7A). Hypoxia reduced the maternal weight gain during pregnancy in both groups and this effect was stronger with the OD mice (Fig 7A). Interestingly, the dam's body weight gain from E 0.5 to E 9.5 was only 2 % in NC-fed under normoxia, 7 % in OD-fed under normoxia, 4 % in NC-fed under hypoxia and 1 % in OD-fed under hypoxia.

The increase of the maternal weight in OD-fed mice was mainly due to an increase in WAT weight both under normoxia and hypoxia as indicated by the WAT to body weight ratio of the dam (Fig.7B). Interestingly, the increase in body weight in OD-fed mice appeared not to be due an increased liver weight (Fig. 7C); yet, a significant reduction in the liver weight to body weight ratio of 19 % was observed when comparing OD-fed mice housed under hypoxia to NC-fed mice housed under hypoxia (Fig. 7C).

When comparing the number of embryos at E 9.5, it was found that OD-fed mice housed under normoxia displayed a significantly reduced embryo number (-22 %, $P \leq 0.05$) than NC-fed mice housed under normoxia (Fig.7D). The reduction in embryo number was not significant when comparing NC-fed mice housed under hypoxia with OD-fed mice housed under hypoxia (Fig. 7D). However, the average embryo weight was similar in all studied groups (Fig. 7E),

indicating that maternal obesity affects embryo number but not embryo weight under normoxia at mid pregnancy.

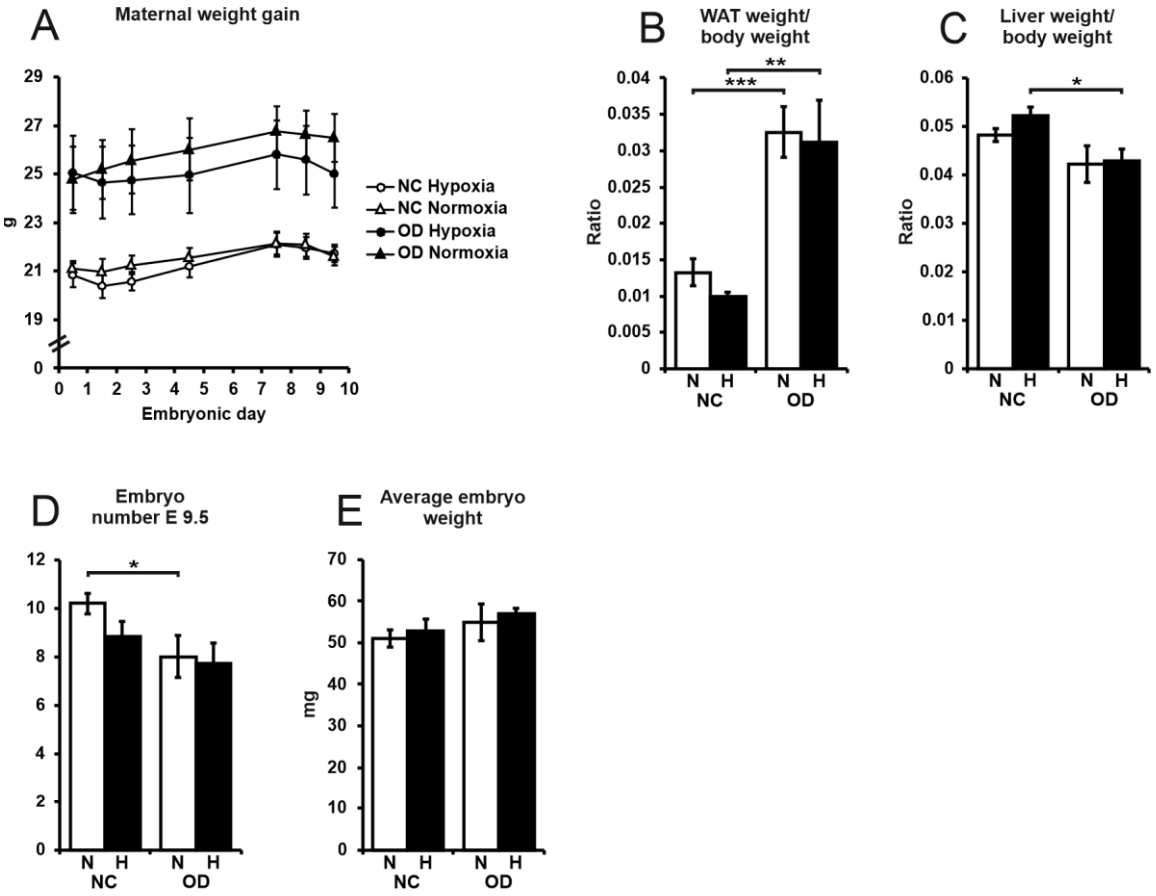

Figure 7: **Effects of OD and hypoxia on maternal and fetal weights**. *Mice were fed either a normal chow (NC) or an obesogenic diet (OD) for 7 weeks before and during pregnancy and were housed under normoxia ($O_2 \approx 21$ %) or normobaric hypoxia ($O_2 = 15$ %) during pregnancy until E 9.5. (n = 5 (N/NC), n = 7 (H/NC) n = 5 (N/OD), n = 7 (H/OD)).* ***A****) Gestational weight gain of dams.* ***B****) Ratio of gonadal WAT to body weight of the dams (total body weight – total embryo weight at E 9.5).* ***C****) Ratio of liver weight to body weight of the dams (total body weight – total embryo weight) at E 9.5.* ***D****) Average embryo number at E 9.5 (n = 51 (N/NC), n = 62 (H/NC), n = 40 (N/OD), n = 54 (H/OD)).* ***E****) Average embryo weight at E 9.5. Data are means ± SEM. Grubbs´s test was used for outliers in A-D). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Student's two-tailed t-test was used for comparison between 2 groups (N-H) in A-D). Abbreviations: N = normoxia, H = hypoxia., WAT = white adipose tissue.*

2.3.4 Effects of OD and hypoxia on maternal metabolic glucose reserves in liver and skeletal muscle

Maternal glucose is an indispensable energy substrate for the fetus during pregnancy and the fetus development is broadly dependent on its metabolic reserves. To determine the effect of OD and hypoxia on maternal metabolic glucose reserves in mid pregnant mice, liver and skeletal muscle glycogen storages were measured. Though hypoxia alone did not affect the levels of glycogen in the livers of pregnant mice, a trend of a 40 % (P = 0.11) decreased glycogen content could be observed upon the combination of OD and hypoxia exposure comparing with OD-fed mice under normoxia(Fig. 8A). While hypoxia had the tendency to lower the maternal muscle glycogen content in both NC- and OD-fed mice by 26 % (P = 0.38) and 42 % (P = 0.06), respectively, OD seemed to increase the muscle glycogen content under normoxia by 26 % (P = 0.30) (Fig. 8B). Serum glucagon levels have been measured as well. The hormone is responsible for maintaining endogenous supply of glucose in fasting stage. Neither hypoxia, nor OD changed significantly the levels of the fasting serum glucagon levels at mid-gestation (Fig. 8C), implying that glucagon is not involved in insulin resistance at this stage of murine pregnancy.

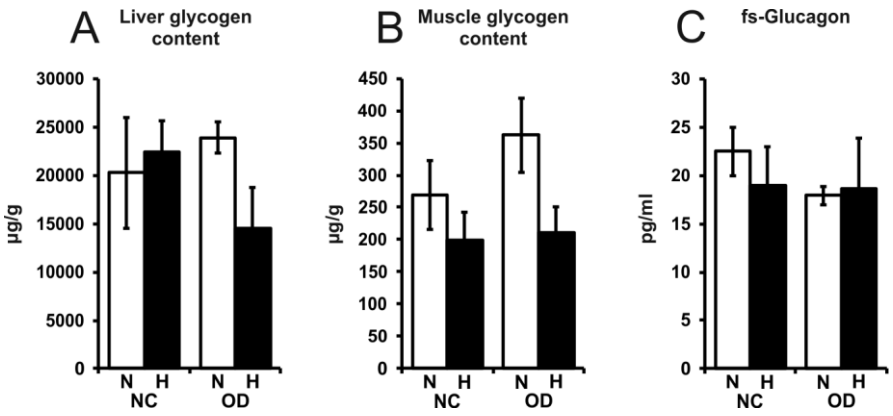

Figure 8: ***Effects of OD and hypoxia on maternal metabolic glucose reserves and glucagon levels:*** *Mice were fed either a normal chow (NC) or an obesogenic diet (OD) for 7 weeks before and during pregnancy, and housed under normoxia (O_2 21 %) or hypoxia (O_2 = 15 %) during pregnancy until E 9.5. (n = 5 (N/NC), n = 7 (H/NC) n = 5 (N/OD), n = 7 (H/OD)).* ***A)*** *Maternal liver glycogen levels at E 9.5, n = 4 (N/NC), n = 7 (H/NC), n = 5 (N/OD), n = 7 (H/OD).* ***B)*** *Maternal skeletal muscle glycogen levels at E 9.5, n = 4 (N/NC), n = 6 (H/NC), n = 5 (N/OD), n = 6 (H/OD).* ***C)*** *Fasting serum glucagon levels at E 9.5, n = 5 (N/NC), n = 6 (H/NC), n = 5 (N/OD), n = 7 (H/OD). Data are means ± SEM. Grubb's test was used for outliers. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Student's two-tailed t-test was used. Abbreviations: N = normoxia, H = hypoxia*

2.3.5 Effects of OD and hypoxia on maternal liver

Diets with a high-calorie content which can cause obesity are known to be a factor for liver dysfunctions like the non-alcoholic liver disease which is characterized by accumulation of triglycerides (Bell 2018). The activation of the hypoxia response has shown some beneficial effects on liver health which was the reason for studying the following liver parameters.

Histological analysis of liver samples obtained at E 9.5 demonstrated a moderate form of hepatic steatosis only in OD-fed mice (40 % steatosis under normoxia and 29 % steatosis under hypoxia) (Fig. 9A). However, statistical significance for steatosis in mice with OD was not reached. All mice fed a NC showed no hepatic steatosis.

Analysis of hepatic triglycerides (TG) was in agreement with the findings of the histological analysis. The liver triglyceride content in both OD groups was significantly elevated compared with NC groups by 21% under normoxia ($P \leq 0.05$) and by 109 % under hypoxia ($P \leq 0.001$). In NC-fed dams hypoxia caused a significant reduction of hepatic TG by 33 % ($P \leq 0.05$). This hypoxia effect was not seen in the OD groups, where was no difference (Fig. 9B). These data indicate that OD induced accumulation of fat in the liver both under normoxia and hypoxia.

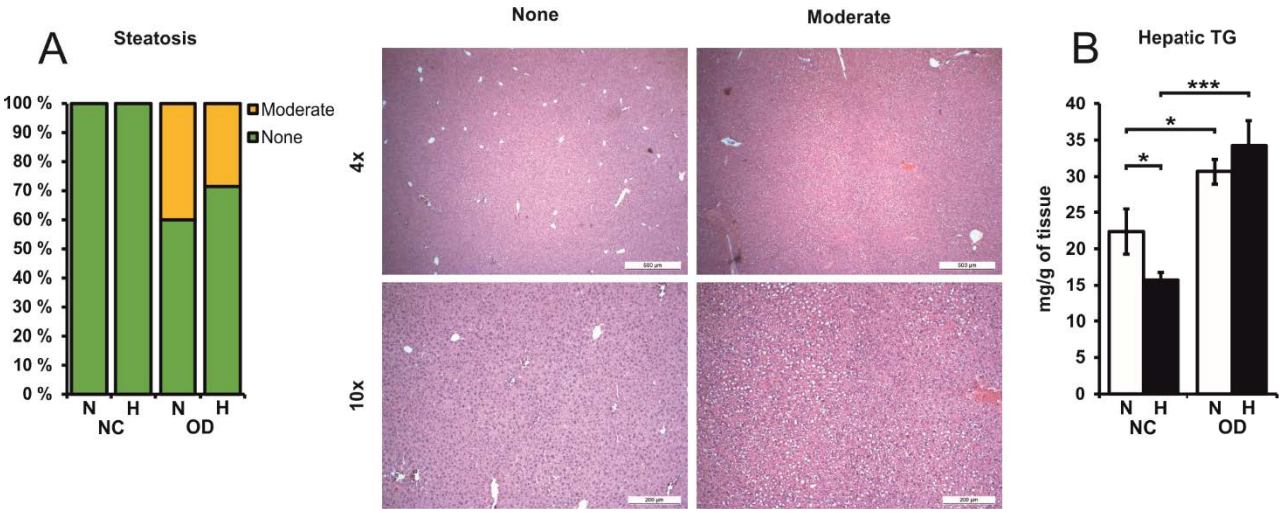

Figure 9: **Effects of OD and hypoxia on maternal liver:** *Mice were fed either a normal chow (NC) or an obesogenic diet (OD) for 7 weeks before and during pregnancy, and housed under normoxia ($O_2 \approx 21$ %) or normobaric hypoxia ($O_2 = 15$ %) during pregnancy until E 9.5. (n = 5 (N/NC), n = 7 (H/NC) n = 5 (N/OD), n = 7 (H/OD)).* ***A)*** *Scoring of hepatic steatosis and H&E stained liver sections at E 9.5. Grading of steatosis: "None" corresponds to scores 0-1 and "Moderate" to 2. 4x corresponds to scale bar = 500 µm and 10x to 200 µm.* ***B)*** *Maternal hepatic TG levels at E 9.5. Fisher's test was used in A). Data are means ± SEM. Grubb's test was used for outliers. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Two-tailed t-test was used in B). Abbreviations: N = normoxia, H = hypoxia.*

2.3.6 The effects of OD and hypoxia on maternal lipid metabolism at mid pregnancy

Obesity in pregnancy has a strong impact on lipid metabolism, elevated serum triglyceride (TG) levels are one result which are also a risk factor for newborn adiposity.(Barbour and Hernandez 2018) The hypoxia response has been reported to alter the lipid metabolism in a beneficial way (Rahtu-Korpela et al. 2014) but not in late pregnancy (Määttä et al. 2018). Therefore, the lipid profile has been studied in mid pregnancy to see if there is a potential effect of hypoxia observable.

The serum free fatty acid (FFA) levels were significantly downregulated from NC to OD under normoxia. Although hypoxia downregulated the levels of FFA in NC-fed mice ($P = 0.06$), the FFA levels in mice receiving the OD and under hypoxia are similar to each other and no difference was observable (Fig. 10A).

The serum TG levels were significantly elevated by 24 % ($P \leq 0.01$) in OD-fed dams housed under normoxia compared with the ones fed a NC. The hypoxia increased significantly the serum TG in NC-fed mice, but no difference was detected between the groups on OD (Fig. 10E).

Diabetic ketoacidosis can occur with a diabetic phenotype, therefore the levels of ketoacids have been investigated. Between NC- and OD-fed groups housed under normoxia no difference in the levels of ketoacids could be observed. NC-fed mice under hypoxia seemed to have reduced amount of ketoacids whereas the combination of OD with hypoxia triggered an increase in ketoacid levels by 83 % ($P \leq 0.05$) in comparison with NC mice kept under hypoxia (Fig. 10B).

The analysis of serum total cholesterol levels showed a significant elevation in the OD-fed groups compared with the NC-fed groups (58 %, $P \leq 0.01$ under normoxia and 32 % under hypoxia, $P \leq 0.001$). However, when comparing the normoxia with the hypoxia group no further difference was observed (Fig. 10C). Similar results were obtained from the HDL-cholesterol analysis. Although it seems likely that hypoxia has an increasing effect on the HDL-cholesterol levels in NC-fed mice, it had a decreasing effect in OD-fed mice. However, both differences were not yet significant (Fig. 10D).

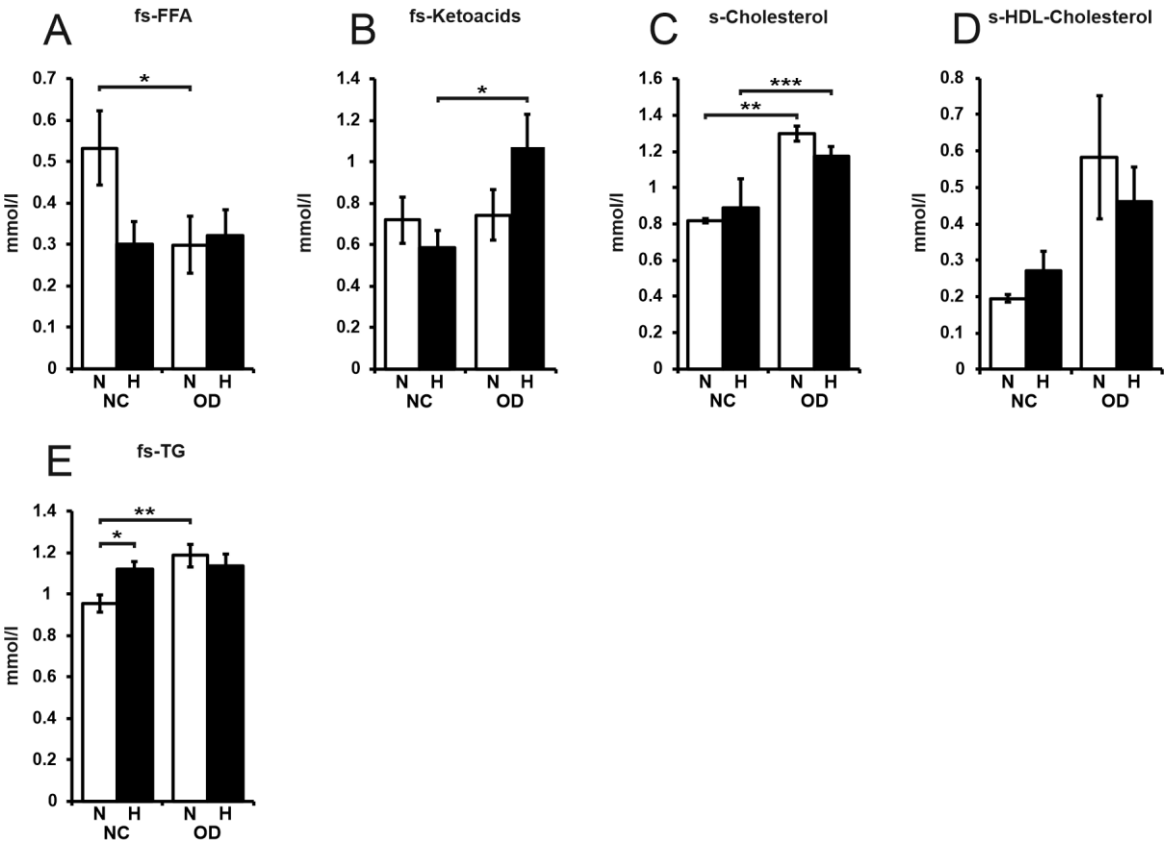

Figure 10: ***The effects of OD and hypoxia on maternal lipid metabolism at mid pregnancy.*** *Mice were fed either a normal chow (NC) or an obesogenic diet (OD) for 8 weeks before and during pregnancy and housed under normoxia ($O_2 \approx 21$ %) or hypoxia ($O_2 = 15$ %) during pregnancy until day E 17.5. (n = 5 (N/NC), n = 5 (H/NC) n = 5 (N/OD), n = 7 (H/OD)).* ***A)*** *Maternal fasting serum free FFA levels at E 9.5.* ***B)*** *Maternal fasting serum ketoacid levels at E 9.5.* ***C)*** *Serum total cholesterol levels at E 9.5.* ***D)*** *Serum HDL cholesterol levels at E 9.5, n = 4 (N/NC), n = 7 (H/NC), n = 5 (N/OD), n = 7 (H/OD).* ***E)*** *Maternal fasting serum TG at E 9.5, n = 5 (N/NC), n = 7 (H/NC), n = 5 (N/OD), n = 7 (H/OD). Data are means ± SEM. Grubb's test was used for outliers. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Two-tailed t-test was used. Abbreviations: N = normoxia, H = hypoxia, HDL = high density lipoprotein, FFA = free fatty acids, TG = triglycerides.*

2.3.7 Effects of OD and hypoxia during pregnancy on mRNA expression of key metabolic genes involved in glucose transport and hypoxia signaling

A mRNA analysis of hepatic tissue, muscle tissue and white adipose tissue (WAT) was done with genes which either play a role in glucose metabolism, hypoxia signaling or inflammation. That is because glucose metabolism is strongly affected by hypoxia and inflammation in WAT is a typical symptom of obesity.

Glucose transporters were downregulated in all analyzed tissues by hypoxia within the NC groups. Hepatic *Glut2* and skeletal muscle *Glut4* mRNA *levels* were downregulated significantly by 24 % ($P \leq 0.01$) and 36 % ($P \leq 0.01$), respectively; the downregulation of *Glut1* mRNA in skeletal muscle tissue did not show significant differences (19 %, $P = 0.1$) (Fig. 11A,D,E). The similar effect was observed within the normoxia groups in which all three analyzed glucose transporters were significantly downregulated in mice fed an OD. Significant differences within the OD-fed groups were not observable in all glucose transporter analyses.

The mRNA analysis of liver type 6-phosphofructokinase (*Pfkl*), an enzyme responsible for the conversion of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate, showed only significant differences in WAT of NC-fed mice housed under hypoxia (Fig. 11B,F,G). Between the NC-fed dams, there was an elevation of *Pkfl* mRNA levels by 44 % from normoxic to hypoxic conditions ($P \leq 0.05$) and within the hypoxia groups a downregulation by 66 % from NC- to OD-fed cohorts ($P \leq 0.001$) (Fig. 11G). There was no difference in *Pfkl* mRNA levels in the livers and muscles of NC-fed and OD-fed mice both under normoxia and hypoxia (Fig. 11B,F).

An important protein for increasing the insulin sensitivity and also a HIF2 target is *Insulin receptor substrate 2* (Irs2) (Taniguchi et al. 2013). Analysis in hepatic tissues showed that OD-fed mice had non-significant lower *Irs2* mRNA levels under normoxia than under hypoxia (40 %, $P = 0.19$) as well compared to NC-fed mice housed under normoxia (44 %, $P = 0.27$) (Fig. 11C).

Pyruvate dehydrogenase kinase 1 (Pdk1) is a major regulator of the oxidative phosphorylation (OXPHOS) and also a key HIF target (Kim et al. 2006). The mRNA analysis in WAT showed no statistical relevant up or downregulation within NC- and OD-fed groups. However, the OD seemed to have a strong impact on *Pdk1* mRNA levels. It was downregulated by 44 % ($P \leq 0.05$) under normoxia and by 50 % ($P = 0.06$) under hypoxia (Fig. 11H).

The chemokine (C-C motif) ligand 2 (Ccl2) is primarily secreted by macrophages and endothelial cells but can also be elaborated by adipocytes. This chemokine promotes the

recruitment of monocytes into inflamed tissue and is therefore a good marker for WAT inflammation (Olefsky and Glass 2010). The analysis of *Ccl2* mRNA levels in WAT revealed lower levels under hypoxia compared with normoxia for mice fed a NC (59 %, $P \leq 0.05$) and within the hypoxia groups the dams on OD showed a strong increase in *Ccl2* mRNA expression (247 %, $P \leq 0.01$) (Fig. 11I).

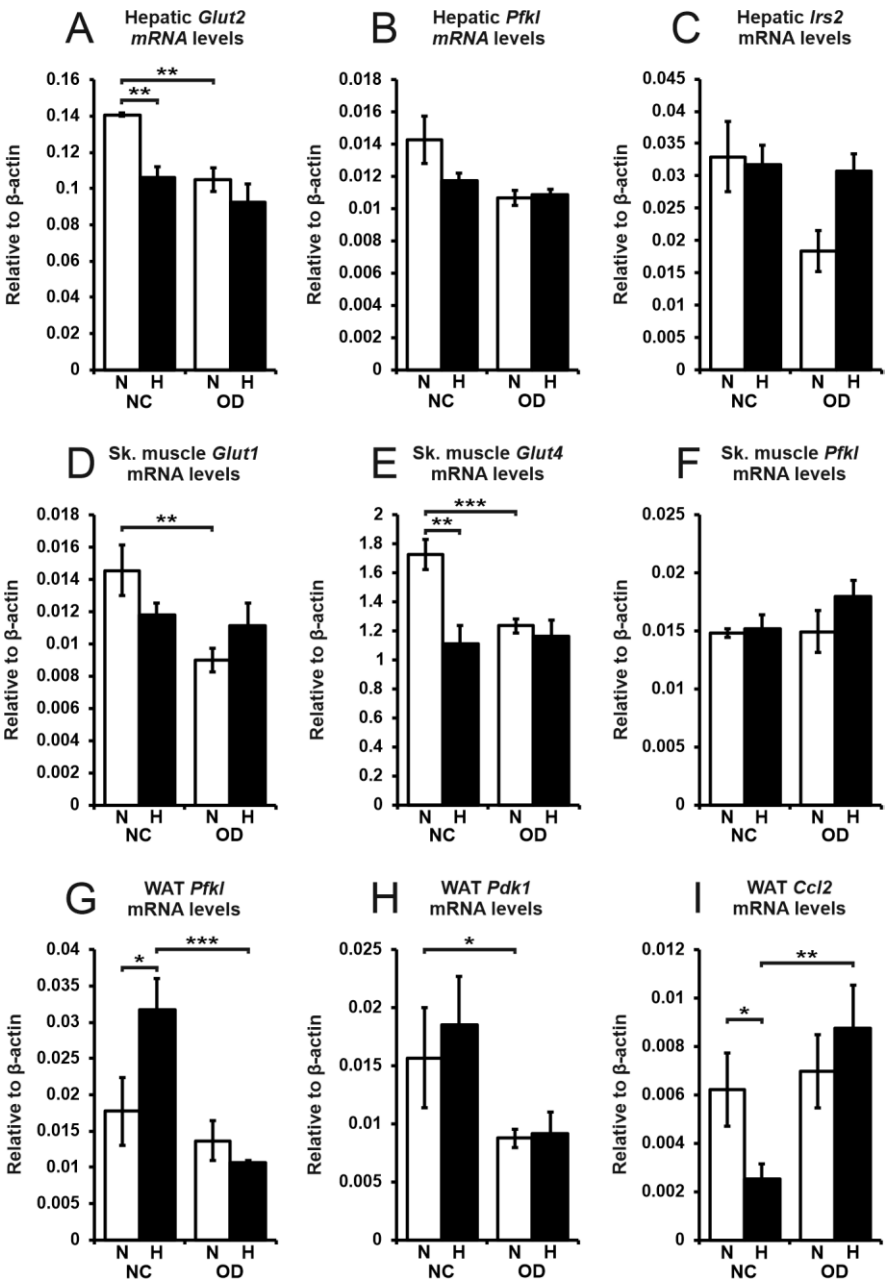

Figure 11: ***Effects of OD and hypoxia during pregnancy on mRNA expression of key metabolic genes involved in glucose transport and hypoxia signaling.*** *Mice were fed either a normal chow (NC) or an obesogenic (OD) for 8 weeks before and during pregnancy and housed under normoxia (O_2 ≈ 21 %) or hypoxia (O_2 = 15 %) during pregnancy until day E 17.5. (n = 5 (N/NC), n = 7 (H/NC) n = 5 (N/OD), n = 7 (H/OD)).* ***A)*** *Maternal hepatic Glut2 mRNA levels at E 9.5, n = 3 (N/NC), n = 7 (H/NC), n = 5 (N/OD), n = 7 (H/OD).* ***B)*** *Maternal hepatic Pfkl mRNA levels at E 9.5, n = 4 (N/NC), n = 7 (H/NC), n = 5 (N/OD), n = 7 (H/OD).* ***C)*** *Maternal hepatic Irs2 mRNA levels at E 9.5, n = 4 (N/NC), n = 7 (H/NC), n = 5 (N/OD), n = 7 (H/OD).* ***D)*** *Maternal skeletal muscle Glut1 mRNA levels at E 9.5, n = 5 (N/NC), n = 7 (H/NC), n = 5 (N/OD), n = 6 (H/OD).* ***E)*** *Maternal skeletal muscle Glut4 mRNA levels at E 9.5.* ***F)*** *Maternal skeletal muscle Pfkl mRNA levels at E 9.5, n = 5 (N/NC), n = 6 (H/NC), n = 5 (N/OD), n = 7 (H/OD).* ***G)*** *Maternal WAT Pfkl mRNA levels at E 9.5.* ***H)*** *Maternal WAT mRNA Pdk1 levels at E 9.5.* ***I)*** *Maternal WAT Ccl2 mRNA levels at E 9.5, n = 5 (N/NC), n = 6 (H/NC), n = 5 (N/OD), n = 7 (H/OD). Data are means ± SEM. Grubb's test was used for outliers. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Two-tailed t-test was used. Abbreviations: N = normoxia, H = hypoxia, WAT = white adipose tissue, Ccl2 = c-c motif chemokine ligand 2, Glut1 = glucose*

transporter 1, Glut2 = glucose transporter 2, Glut4 = glucose transporter 4, Irs2 = insulin receptor substrate, Pdk1 = pyruvate dehydrogenase lipoamide kinase isozyme 1, Pfkl = phosphofructokinase, liver type.

2.3.8 The impact of maternal obesity and hypoxia in late pregnancy

To study the effects of an OD and hypoxia on the diabetic phenotype in late pregnancy, the same experimental setup was followed as before. In brief, 4-month-old mice were control-fed with NC and an OD for 7 weeks before mating and during pregnancy. Based on both homeostatic model assessment-insulin resistance (HOMA-IR) and body weight, the control group (HOMA-IR ~12,9 body weight 23,1 g) and the diabetic group (HOMA-IR ~17.4, body weight 27,8 g) were formed. After mating and detection of the vaginal plug the mice were further divided into a normoxia and hypoxia (15% O_2) group each. This resulted in four groups: 1) NC-fed normoxia, 2) NC-fed under hypoxia, 3) OD-fed under normoxia, and 4) OD-fed under hypoxia. A glucose tolerance test was performed on mice at day E 17.5 after vaginal plug detection and then the mice were sacrificed. Although all mice initially had vaginal plugs, there were non-pregnant mice (N/NC = 3; H/NC = 1; N/OD = 4; H/OD = 3) which were subsequently taken out of all analyses and this resulted in changed starting average body weight and HOMA-IR in the groups, especially in the OD-normoxia (~25 g, ~11.8 HOMA-IR) and OD-hypoxia groups (~30 g, ~15.5 HOMA-IR).

2.3.9 Effects of OD and hypoxia on glucose tolerance and insulin sensitivity in late pregnancy

There was no significant difference in glucose tolerance of mice fed NC under normoxia or hypoxia at day E 17.5 (Fig. 12A, B) whereas OD caused a trend of a reduced glucose tolerance under both normoxia and hypoxia (Fig. 12A, B). Although dams on OD housed under hypoxia had a clear tendency for an improved glucose tolerance at day E 17.5 compared with OD dams housed under normoxia, a statistical significance was not reached (Fig. 12A, B). Also, the fasting serum insulin levels did not show a statistical significance among the groups (Fig. 12C); however, in OD groups, insulin levels increased by 53 % ($P = 0.1$) under normoxia and by 81 % ($P = 0.09$) under hypoxia. Similarly, while HOMA-IR did not differ between normoxia and hypoxia in NC groups (Fig. 12D), OD increased the HOMA-IR under normoxia and hypoxia; however, only the increase under normoxia was significant (Fig. 12D). Surprisingly, hypoxia alone exerted a negligible effect on hemoglobin (Hb) levels (Fig. 12E); OD significantly decreased Hb levels in dams housed under both normoxia and hypoxia by about 12 % (NC: $P \leq 0.05$; OD: $P \leq 0.01$) (Fig. 12E). Lactate levels were higher under hypoxia in the

NC groups and lower in the OD groups. However, there were no significant differences within those groups (Fig. 12F). OD had only an effect in the normoxia groups on the lactate level, within the hypoxia groups no changes were observable.

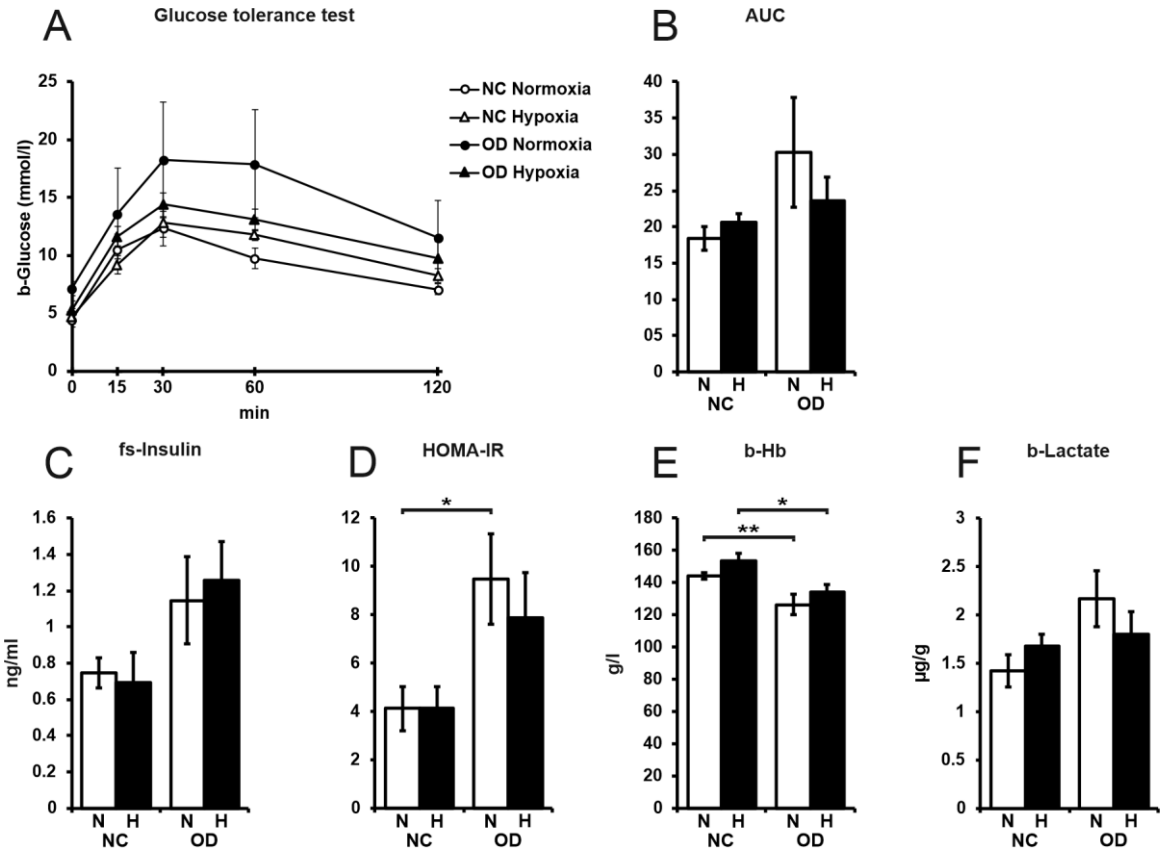

Figure 12: ***Effects of OD and hypoxia on glucose tolerance and insulin sensitivity in late pregnancy.*** *Mice were fed either a normal chow (NC) or an obesogenic diet (OD) for 8 weeks before and during pregnancy and housed under normoxia ($O_2 \approx 21$ %) or hypoxia ($O_2 = 15$ %) during pregnancy until day E 17.5. (n = 6 (N/NC), n = 4 (H/NC) n = 4 (N/OD), n = 6 (H/OD)).* ***A)*** *Glucose tolerance test at E 17.5. 0-minute value was determined after 4h fasting.* ***B)*** *Area under the curve of glucose tolerance test at E 17.5.* ***C)*** *Fasting serum insulin levels at E 17.5.* ***D)*** *HOMA-IR levels at E 17.5.* ***E)*** *Maternal blood Hb levels at E 17.5.* ***F)*** *Maternal blood lactate levels at E17.5. Data are means ± SEM. Grubb's test was used for outliers. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Two-tailed t-test was used. Abbreviations: N = normoxia, H = hypoxia, AUC = area under the curve, HOMA-IR = homeostatic model assessment for insulin resistance, Hb = hemoglobin.*

During pregnancy, the maternal weight gain was increased though with different rates (Fig. 13A). In the NC group, the weight gain was 42 % under normoxia and 53 % under hypoxia. The maternal weigh gain of dams on OD was 35 % under normoxia and 25 % under hypoxia (Fig. 13A). However, all mice had a similar end-point body weight (Fig. 13B). The ratio of the WAT weight to the maternal body weight was significantly increased in dams on OD (Fig. 13C). Although the effect of hypoxia on the adiposity was not significant, there was a trend for a reduced WAT/body weight ratio in dams on OD ($P = 0.27$) (Fig. 13C). The OD, but not hypoxia, affected also the liver to body weight ratio with a significant lower ratio in the OD groups compared with NC groups (Fig. 13D). There was no difference in embryo number as well as in average placenta weight of all studied groups of mice (Fig. 13E, G). As expected, OD had a significant impact on the average birth weight of the embryos and decreased it by 25 % ($P \leq 0.01$) under normoxia and by 13 % ($P \leq 0.01$) under hypoxia (Fig. 13F).

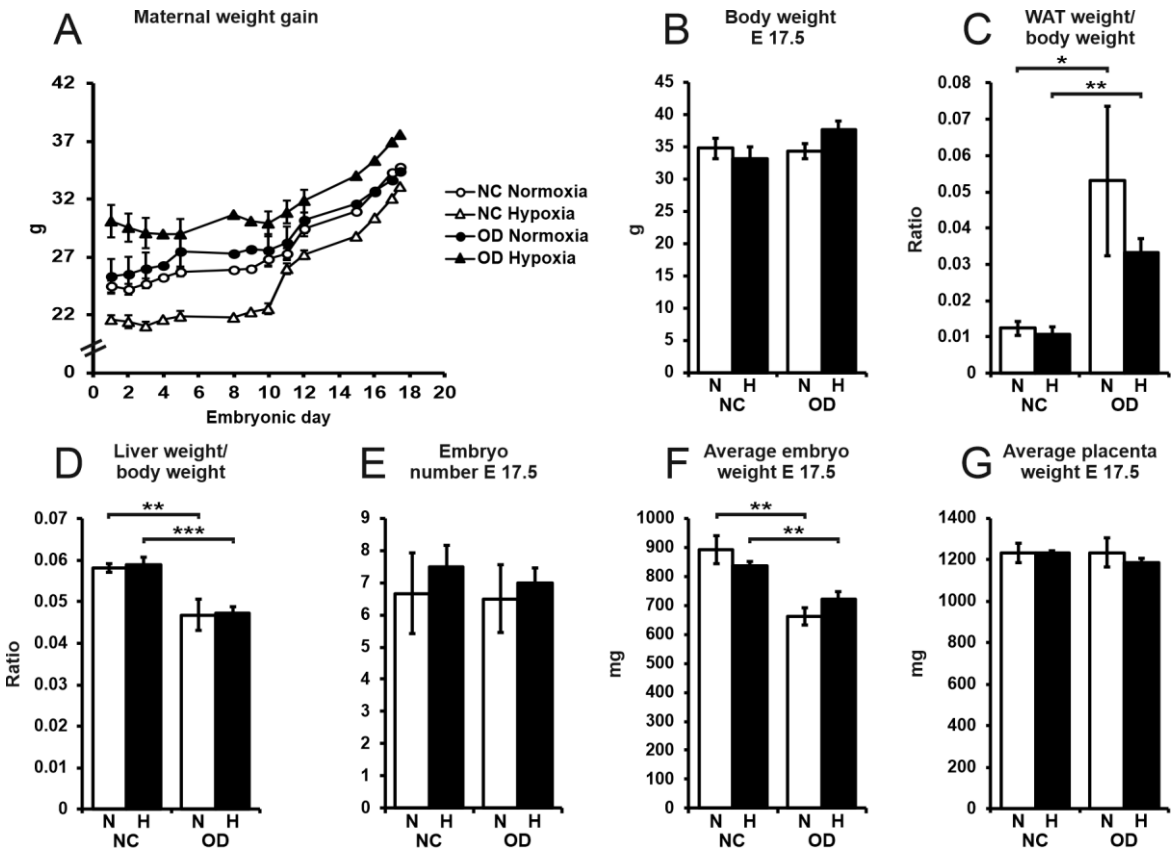

Figure 13: ***Effects of OD and hypoxia on maternal and fetal weights in late pregnancy.*** *Mice were fed either a normal chow (NC) or an obesogenic diet (OD) for 8 weeks before and during pregnancy and housed under normoxia ($O_2 \approx 21\%$) or hypoxia ($O_2 = 15\%$) during pregnancy until day E 17.5. (n = 6 (N/NC), n = 4 (H/NC) n = 4 (N/OD), n = 6 (H/OD)).* ***A)*** *Gestational weight gain of dams.* ***B)*** *Endpoint body weight pregnant mice at E 17.5.* ***C)*** *Ratio of gonadal WAT to body weight of the dam (total body weight – total embryo weight-total placental weight) at E 17.5.* ***D)*** *Ratio of maternal liver weight to body weight of the dam (total body weight – total embryo weight-total placental weight) at E 17.5.* ***E)*** *Average embryo number at E 17.5.* ***F)*** *Average embryo weight at 17.5.* ***G)*** *Average placenta weight at E 17.5. Data are means ± SEM. Grubb's test was used for outliers. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Two-tailed t-test was used. Abbreviations N = normoxia, H = hypoxia, WAT = white adipose tissue.*

2.4 Discussion

Chronic hypoxia at high altitude (>2500m) lowers the birth weight by causing intrauterine growth restriction (IUGR) and it has the second greatest influence on birth weight right after gestational age (G. M. Jensen and Moore 1997)(L.G., S.M., and C.G. 2011)(Soria et al. 2013)(Julian et al. 2007)(E. Krampl 2002). Due to its impact on reducing fasting glucose values, especially in the second half of pregnancy, the energy availability for the fetus is much lower (Elisabeth Krampl et al. 2001). In contrast, gestational diabetes mellitus (GDM) causes hyperglycemia in many women which leads to greater supply of the main macronutrient glucose for the growing child and causes fetal overgrowth (Pettitt et al. 1980). The aim of this study was to investigate if hypoxia can ameliorate the gestational diabetes symptoms and inflammatory response thus lowering the risks of complications for obese mothers with gestational diabetes and their newborns.

We used mouse model in our study and preconditioned the mice for developing GDM by feeding them an obesogenic diet (OD), thus simulating the most significant risk factor, which is a state of overweight and obesity (Zhang and Ning 2011). By comparing blood glucose and HOMA-IR values of the cohorts (Table 9) it appeared that the mice of the OD-group are in a more "diabetic-like state" compared to NC-fed mice under normoxic conditions. However, those mice were not "diagnosed" with GDM by using glucose tolerance test (GTT), like GDM would be diagnosed in pregnant women according to the standards of the "International Association of Diabetes and Pregnancy Study Groups" (Boyd E. Metzger 2010). Therefore, it is not entirely correct to refer to a GDM like phenotype in these mice, per definition. Still the previous mentioned elevated glucose and HOMA-IR levels indicate a more diabetic-like phenotype. In this study, hypoxia failed to significantly improve these values in NC-fed mice in contrast to previous data (Määttä et al. 2018). However, the OD-fed mice showed a less strong increase of glucose and HOMA-IR levels under hypoxia compared to normoxia. though the difference was not significant. Thus, further investigation is needed in order to identify the effect of hypoxia and obesity on pregnant mice (Fig. 7A). Our findings show that mice kept under hypoxic conditions had a non-significant lower amount of WAT compared to normoxic mice. This failure in increasing the WAT amount in the first trimester is most likely due to inefficient glucose metabolism inducing OXPHOS and resemble results from previous experiments (Fig. 7B) (Määttä et al. 2018). Comparison of the average embryo weight between the groups showed an effect of neither hypoxia nor obesity, like it is observed in humans. All embryos had approximately the same average weight (Fig. 7E); this result is in line with a

common finding for embryos from mothers in OD-fed mouse models (Christians et al. 2019). Similarly, according to a meta-study investigating the link between fetal overgrowth and obesogenic diet in rodent models, there was no observable effect of the diet on fetal growth in 45% of the studies (Christians et al. 2019). Another explanation for no change in embryo weight could be that in many cases GDM is developed during late pregnancy. The samples in this study were taken collected during mid-pregnancy (E9.5) which means that the developing insulin resistance and related increased glucose availability did not affect the growth of the pups yet. We could also see a reduced number of embryos of dams received the OD compared to NC-fed under normoxia as well as a trend under hypoxia (Fig. 7D). This might be explained by a reduced number of ovulated and fertilized oocytes which was found in obese mice models (Finger et al. 2015). Reduced fertility is an issue which affects many obese humans as well (Hart and Hunter 2018).

Glycogen is a "glucose storage" polymer and it is stored mainly in the liver and skeletal muscle. Its main function is to supply glucose to the bloodstream during periods of fasting and/or muscle contraction. Because we have seen that both OD and hypoxia have an effect on maternal glucose and insulin levels (Table 1 and Fig. 1), we next studied the amount of glycogen in the liver and skeletal muscle of mid pregnant mice (Fig. 8A,B).

In the liver, the OD-fed dams kept in hypoxic conditions showed reduced glycogen levels while all other studied groups of mice have similar glycogen levels (Fig. 8A). These data imply that neither hypoxia nor the insulin resistance developed through OD alone could affect liver glycogen content; the combination of both factors which probably results in a higher energy demand due to hypoxia and reduced glucose uptake into the tissue because of insulin resistance led to the reduced, though not significant, values of liver glycogen concentrations.

Glycogen concentration in the muscles was reduced in hypoxic NC- and OD-fed mice which might be due to the increased energy demand in lower oxygen concentrations. Because of the upregulated glycolytic metabolism and OXPHOS suppression under hypoxic conditions the amount of ATP generated by each glycogen molecule is 18 times lower. The main product of increased glycolysis is lactate which is acidifying the cell. HIF-target genes promoting the reuse and clearance of lactate by activating the Cori cycle, resulting in gluconeogenesis. Therefore, the dams do not have enough energy left to fill up their reservoirs in the muscles. The amount of glycogen in the OD mice kept under normoxic conditions is higher (Fig. 8B) which is most likely due to the higher amount of available energy, even though it would be assumed that

through altered insulin signaling the glucose uptake which is needed for glycogen synthesis is reduced.

Glucagon is a peptide hormone produced by alpha cells of the pancreas is responsible for maintaining the endogenous supply of glucose in fasting stage by causing the liver to convert stored glycogen onto glucose. Our analysis of fasting serum glucagon levels at mid-gestation could not show any significant difference between our investigated groups (Fig. 8C). Those results imply that glucagon is not involved in insulin resistance of murine pregnancy at this stage of pregnancy.

Non-alcoholic fatty liver disease (NAFLD) is a constantly growing health problem because of the increasing calorie uptake especially in industrialized countries. The disease is characterized by the accumulation of triglycerides (TG) in the liver as well as by systemic metabolic symptoms. Untreated NAFLD leads to liver steatosis, cirrhosis and even hepatocellular carcinoma (Diehl and Day 2017). In previous studies it could be shown that the inhibition of HIF-P4H-2, the major regulator of HIFαs, which leads to activation of hypoxia response in normoxic conditions, protects mice from high fatty liver associated metabolic dysfunction and hepatic steatosis (Rahtu-Korpela et al. 2014). To check the extend of hepatic steatosis during pregnancy caused by pre-pregnancy OD and/or hypoxia exposure during pregnancy, we studied hepatic steatosis (Fig. 9A,B). As expected, an increase of steatosis in mice obtained the OD could be seen by histological evaluation and the obese mice under hypoxia showed less steatosis than the ones under normoxia (Fig. 9A). The hepatic TG were significant elevated in the OD mice even though there was no difference between normoxia and hypoxia groups (Fig. 9B). In H/NC group hepatic TG levels were lower compared to N/NC group (Fig. 9B) and this could be due to the protective effect of activated hypoxia signaling (Rahtu-Korpela, 2014, Laitakari 2019, Laitakari 2020). It is important to mention that the mice in the present study were in total only 6-8 weeks on an OD diet. This time period is normally too short to develop NAFLD or more severe symptoms in the liver like in the mice of the other experiments.

It has been shown that GDM and obesity in pregnancy increase the risk for delivering infants which are significantly heavier for their gestational age. Those infants show increased adiposity at birth. The same effect can also be observed in obese mothers with no GDM like increased insulin resistance and in women with GDM but glycemic control. Higher maternal TG levels and therefore elevated amounts of FFA from early pregnancy on are responsible for this effect (Barbour and Hernandez 2018). Hif-P4H-2 hypomorphic mice (Hyvarinen 2010) that show

stabilization of HIFαs i.e activation of hypoxia response appear to alter the lipid metabolism in a beneficial way (Rahtu-Korpela 2014) but not during late pregnancy (Määttää). Therefore, in this study we also determined the maternal lipid profile to check if the OD, hypoxia or combination of both can alter the maternal lipids at mid-pregnancy. N/OD dams showed a significant increase of TG compared to mice receiving the NC in normoxia which is half the increase that can be seen in humans. Interestingly, there is a significant increase of TG in H/NC mice compared to N/NC at mid-pregnancy (Fig.10E) but such a difference could not be seen at late pregnancy (Määttä et al. 2018) nor in non-pregnant mice with activated hypoxia response (Rahtu-Korpela et al. 2014). The pathophysiological changes which occur during GDM and in pregnant obese women i.e. increased TG levels come with elevated FFA levels can also be observed in the mice on OD compared to NC-fed mice. This was not the case in our study. Our results show that N/NC mice had the highest FFA concentration of all studied groups. The other groups all show similar concentrations of FFA and TG (Fig.10A).

Diabetic ketoacidosis is a leading cause of fetal death (Schneider et al. 2003). It is a serious metabolic complication of diabetes characterized by hyperglycemia, metabolic acidosis and ketone body concentration (Kitabchi et al. 2009)(Kamalakannan et al. 2003). However, it is a very rare complication in diabetic women during pregnancy with a prevalence of 1-2 % (Parker and Conway 2007) and even lower in women with GDM (Bedalov and Balasubramanyam 1997)(Pitteloud et al. 1998). During late pregnancy, the serum ketone bodies are increased compared to nonpregnancy in diabetic mothers and in fasting (Herrera 2002). The ketone bodies can be used both by the mother as alternative glucose substrate and by the fetus as a fuel for its metabolism and/or lipogenic substrates (Herrera E, 2002). The data of the present study shows elevated levels of ketoacids in H/OD group mice at mid-pregnancy (Fig. 10B). It seems like that the combination of hypoxia and OD is triggering the production of ketoacids at this stage of murine pregnancy. However, if this increase is beneficial and physiologically relevant, is not clear yet because blood pH was not analyzed, and pathological symptoms of the embryos were not observed.

The homeostasis of cholesterol is crucial for maintenance of feto-placental endothelial function. In GDM processes are upregulated which can cope with increased cholesterol levels produced in placental endothelial cells. Those mechanisms remove cholesterol from the feto-placental circulation in order to avoid the formation of pre-atherosclerotic lesions (Scholler et al. 2012)(Sun et al. 2018). In previous experiments it could been shown that the activation of the hypoxia response could lower the serum cholesterol levels (Rahtu-Korpela et al. 2014).

However, our current results did not show any differences in serum cholesterol levels upon hypoxia (Fig. 10C,D). However, a significant elevation in both OD-fed groups compared to NC-fed groups in serum total cholesterol and a trend for increased serum HDL-cholesterol. These results suggest that an obesogenic diet is clearly affecting cholesterol availability during mid pregnancy while hypoxia did not has an effect. The combination of OD and hypoxia on maternal lipid metabolism could not show a beneficial effect by lowering serum lipid values in the investigated parameters.

Glucose transporters are the rate limiting step for glucose utilization in various tissues. In healthy individuals, insulin is increasing glucose uptake in striated muscle by recruiting GLUT4 (not GLUT1) from an intracellular pool to the cell membrane. In diabetic individuals this recruiting process is impaired (Klip et al. 1992). Similar impairment is occurring in women with GDM. The mRNA levels for GLUT4 is reported to be increased in muscle tissue of Hif-p4h-2 hypomorphic mice that show stabilization of HIFαs (Rahtu-Korpela et al. 2014). In our study, the mRNA analysis of skeletal muscle tissue showed a significant reduction in *GLUT4* mRNA expression in H/NC mice compared to normoxic mice on NC and no differences in *GLUT4* mRNA expression within the OD groups (Fig. 11E). Also, for mRNA levels of *GLUT1* in the skeletal muscle we could not observe a significant difference between normoxia and hypoxia groups (Fig. 11D). The analyzes of hepatic tissue showed no alterations in mRNA expression in response to hypoxia except for a decrease in *GLUT2* in H/NC mice (Fig. 11A). The obesogenic diet affected the observed mRNA expression in the liver and muscle where the expression levels of GLUT1, GLUT2 and GLUT4 were lower within the normoxia groups, thus indicating a more diabetic-like phenotype (Fig. 11A,D,E). Taken the mRNA analyses together we found that hypoxia is not capable of increasing the expression levels of glucose transporters at mid pregnancy like it is the case for non-pregnant mice. Interestingly, the mRNA level of the well-known HIF1α target *Pfkl* in the muscle was unaffected by hypoxia in both dietary groups (Fig. 1F). Lower hepatic *Pfkl* levels, non-significant though, in N/OD compared to N/NC group (Fig. 11B) may be a sign of a reduced glycolysis. This effect can also be due to reduced glycogen uptake in the tissue because of higher insulin resistance. H/OD group also showed lowered *Pfkl* levels compared to N/NC dams. This effect is the opposite of previous findings in late pregnancy, where dams kept in hypoxic conditions had higher *Pfkl* expression levels (Määttä et al. 2018). In WAT is a significant increase of *Pfkl* due to hypoxia in the NC groups. It is also the only organ analyzed which showed this response to hypoxic conditions (Fig. 11G). It is unclear why we see this effect only in WAT and not in liver or muscle. A possible

explanation could be that the duration of the mice living in hypoxic conditions was too short to obtain the full hypoxia response on mRNA in all organs. While comparing the hypoxia groups with each other the OD neglected this increasing effect of *Pfkl* completely.

Another HIF target gene which act as a key protein in increasing insulin sensitivity is hepatic *Irs2* (Taniguchi et al. 2013). It was downregulated in the N/OD mice (Fig. 11C), which may be caused by the decreased insulin sensitivity in our diabetic-like mice. OD dams kept in hypoxic conditions seemed to be protected from this decrease since those mice showed similar expression levels as both NC groups (Fig. 11C). It looks like that hypoxia is increasing the gene expression to a certain level and thus compensates for the effects of OD.

Pdk1 mRNA levels are affected by OD but not by hypoxia in WAT (Fig. 11H) implying that OD does not actively downregulate the OXPHOS. Obesity is linked with chronic low-grade inflammation which predisposes for insulin resistance (Harford et al. 2011)(Fuentes et al. 2013). Therefore, we examined the mRNA levels of *Ccl2* in WAT (Fig. 11I); *Ccl2* is a chemokine that promotes the recruitment of macrophages into inflamed tissue (Olefsky and Glass 2010). *Ccl2* mRNA levels were significant downregulated under hypoxia in NC group (Fig. 11I). This result is in agreement with findings that chronic hypoxia can reduce macrophage aggregates around adipocytes (Van Den Borst et al. 2013) and suggests that chronic hypoxia has an anti-inflammatory effect in WAT of NC-fed mice. This effect cannot be seen within both groups received OD. Hypoxia seems to even increase the mRNA expression of this inflammatory marker.

In the second part of this study we checked the effects of an OD and hypoxia on the diabetic phenotype in late pregnancy (E 17.5) using the same experimental setup. Those mice underwent a glucose tolerance test (GTT) before sacrifice. Our NC-fed dams under hypoxia did not show improved glucose tolerance as it could have been shown in a previous study (Määttä et al. 2018). Hypoxia seems to increase the glucose tolerance within the OD groups, but it is not significant. Mice kept on an OD despite normoxia or hypoxia have further decreased glucose tolerance in late pregnancy compared with dams received NC (Fig. 12A). This further decreased glucose tolerance is one of the hallmarks of GDM and can also be seen in pregnant obese women (McIntyre et al. 2019). This trend in the GTT goes along with the insulin levels and the HOMA-IR (Fig 12C,D). There we also seen in the OD groups elevated values compared to the NC dams. Considering the effect of hypoxia on those parameters, there is no difference observable compared to normoxic conditions (Fig. 12C,D). However, those findings could not reach

statistical significance therefore further investigation is needed. We also see significant decreased hemoglobin levels dams received OD (Fig. 12E) which is the opposite of that what we can see in humans (Elmugabil et al. 2017). Hypoxia itself affects hemoglobin levels by inducing erythropoiesis through elevated EPO secretion (Wang and Semenza 1993) Our dams kept in hypoxic conditions had a small non-significant increase in hemoglobin which indicates hypoxia response (Fig. 12E).

The comparison of the maternal weight gain between our groups is affected by different starting weights of the dams kept in the same diet group but under hypoxic or normoxic conditions (Fig. 13A/B). Several mice had to be taken out of the experiment due to false pregnancy which then affected the average starting weight of our groups. For more accurate values the measuring of maternal weight gain should be repeated. As expected, the ratio of WAT was significant higher in both OD groups compared with NC groups. However, the increase of the H/OD group was not as high as the one of the N/OD group. This shows the less efficient glucose metabolism in hypoxic conditions, which results in lower increase of WAT (Fig. 13C). In this late pregnancy experiment (E 17.5) we did not see any difference in embryo number per dam (Fig. 13E) compared to our mid-pregnancy experiment we see this reduced embryo number in mice received OD. Most likely not only obesity plays in the fertilization process a role, other factors like stress, animal number and hormones are also involved.

2.5 Conclusions

Taking all the results from this mid-pregnancy experiments together there was no clear effect of hypoxia on diabetic like mice observable. Even though some relevant parameters were altered between N/OD and H/OD group like decreased glycogen values in the liver and muscle and elevated *Irs2* mRNA levels. However, those values could not reach significance. The same applies to the results from the late-pregnancy study where we did not observe significant increase in glucose tolerance as well as decreased HOMA-IR values in the H/OD mice compared to the N/OD.

Nevertheless, strong effects of the obesogenic diet in various analyzed parameters during mid-pregnancy could be observed in this study. The OD affected many physiological aspects in an unfavorable way. It had a strong impact on glucose metabolism by decreasing glucose tolerance and increased insulin values as well as on the lipid metabolism. Mice fed with OD showed for e.g. elevated fs-TG levels, hepatic TG levels and cholesterol levels in the state mid-pregnancy. During late-pregnancy similar effects of the OD became evident like elevated HOMA-IR values in addition to a higher WAT to bodyweight ratio.

An important limitation that needs to be considered when interpreting these results is that the group sizes and other parameters were not as consistent as anticipated. This was due to the fact of mating problems with the corresponding mice during the late-pregnancy study. This makes comparison and interpretation of the present results more difficult. Part of the experiments should be repeated, and further research needs to be done in order to get a clearer insight into possible links between the described conditions and a better understanding of the underlying molecular mechanisms.

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2.7 Annexes

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Declaration

I hereby certify that I composed the present thesis with the topic:

“The effects of hypoxia on gestational diabetes mellitus in mice”

independently and that I have used no other sources other than those indicated. The text passages which are taken from other works in wording or meaning I have identified in each individual case by stating the source.

I further hereby declare that I have completed my academic work in line with the principles of good scholarly and scientific practice and in accordance with the valid “Article of the University of Ulm for Ensuring Good Scientific Practice”

Ulm, 31.03.2020_____

(Florian Mohr)